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14. ABSTRACT
Breast cancer (BC) has a predilection for bone metastases. While the mechanism for directional metastasis is unknown, the bone microenvironment likely provides a fertile soil for metastatic BC cells. Besides affecting osteoblast (OB) and osteoclast (OC) properties, we have evidence that metastatic BC cells further create a unique bone niche by co-opting osteoblasts to increase production of inflammatory cytokines that may be chemoattractants, growth, or maintenance factors for cancer cells or OCs. Our purpose is to determine how OB-derived cytokines influence BC metastases to bone. Goals include investigating the production of OB-derived cytokines in response to BC cells or their conditioned medium (CM), the production of bone-derived cytokines in response to BC cells in vivo, the presence of functional cytokine receptors on OBs and BC cells, and the chemoattractant effect of OB-derived cytokines on BC metastasis. Using murine osteoblasts and human non-osteoblast variants, we found that BC CM treatment increased osteoblast-derived cytokine secretion of IL-6, KC, VEGF, MIP-2, and MCP-1. Maximum induction of osteoblast-derived cytokine secretion occurred with 20 day old cells. Human metastatic BC cells produce very small quantities of MCP-1. When osteoblasts and non-osteoblasts were placed in a co-culture system, non-osteoblast-derived cytokine production decreased significantly from baseline amounts. Murine bone cell-derived cytokine production increased when human metastatic cancer cells were present in the bone microenvironment.

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INTRODUCTION

Breast cancer preferentially metastasizes the ends of long bones in humans[1]. The mechanism for preferential metastasis is unknown, but it is likely that bone provides a hospitable environment for breast cancer cell colonization and survival[2].

Bone resorbing osteoclasts initiate bone remodeling by the excavation of an erosion cavity in the bone matrix[3]. Bone depositing osteoblasts migrate to the site of remodeling and synthesize osteoid matrix to fill in the erosion cavity, with no net bone loss or gain[3]. Bone metastatic breast cancer cells disrupt bone remodeling to favor of bone resorption. Osteoclasts are constitutively activated, resulting in osteolytic lesions that cause bone pain and hypercalcemia[4]. Osteoblasts cease depositing bone and undergo apoptosis[5]. Current therapies utilize bisphosphonates to block osteoclast function and slow osteolytic lesion progression[6]. Lesions already present, however, do not heal[7]. Research has focused on breast cancer cell-derived cytokine production as key to understanding preferential bone metastasis[8]. While they may have a supporting role, we have evidence that bone metastatic breast cancer cells direct *osteoblasts* to produce inflammatory cytokines that may indirectly be chemoattractants, growth, or maintenance factors for the cancer cells.

For my ongoing research, I hypothesize that ***osteoblast*-derived cytokines are increased in the presence of metastatic breast cancer cells and act as chemoattractants, growth, and maintenance factors for them.** The aims of this proposal are: 1) To determine how *osteoblast*-derived inflammatory cytokine production by MC3T3-E1 osteoblasts is altered in response to co-culture or conditioned media of bone metastatic MDA-MB-231 breast cancer cell variants. 2) To determine how bone-derived inflammatory cytokine production is altered in response to breast cancer cells *in vivo*. 3) To determine if osteoblasts and breast cancer cells have receptors and can respond to *osteoblast*-derived inflammatory cytokines.

BODY

As listed at the conclusion of last year's annual report, several key research accomplishments were determined:

- Osteoblasts are an important source of KC, MCP-1, IL-6, MIP-2, and VEGF in the vicious cycle of breast cancer bone metastasis. MCP-1 is a particular cytokine of interest in this group because it was not expressed by breast cancer cell conditioned medium or osteoblasts treated with non-metastatic cell conditioned medium.
- Osteoblast-derived cytokine production in response to MDA-231 variant conditioned medium was found to be dose-dependent.
- Maximum induction of osteoblast-derived cytokine secretion was found to occur in more differentiated (20 day old) cells. This suggests that the stage of osteoblast differentiation is important in determining the osteoblast response to metastatic breast cancer cells.
- It was discovered that treatment with the conditioned medium of a MDA-231 bone seeking variant further enhanced osteoblast-derived cytokine production at day 20.

- Finally, data suggest that osteoblast-derived cytokine production is necessary, but not sufficient, for the survival of bone metastatic breast cancer.

Since then, much work has been done contributing to the progress of this fellowship.

Task 1. **To determine how *osteoblast*-derived inflammatory cytokine production by MC3T3-E1 osteoblasts is altered in response to co-culture or CM of bone metastatic MDA-MB-231 breast cancer cell variants.**

Task 1a: Collect culture media from MC3T3-E1 (at 4 days, 14 days, and 20 days), hTERT-HME1, MDA-MB-231P, MDA-MB-231BO, MDA-MB-231BR, and BRMS1 transfected-MDA-MB-231 cells at 24 hours. Perform a RayBio® species-specific antibody array. In addition to progress documented in the previous annual report, conditioned media (CM) was collected at 24 hours from the following additional cell lines, representing various tumorigenic and metastatic potentials, species, and cell origins. Conditioned media (CM) was obtained by growing cells to confluence and incubating them in serum-free media for 24 hours. CM was stored at >-20°C until used.

- MDA-MB-468P human non-metastatic breast cancer cells
- MCF-10A human non-metastatic breast cancer cells, a non-tumorigenic cell line derived from a female with fibercystic disease
- HBL-100 human non-metastatic breast cancer cells, a tumorigenic cell line obtained from an early lactation sample of human milk. There was no evidence of a breast tumor in the female donor.
- HC-11 mouse non-tumorigenic, non-metastatic immortalized mammary epithelial cells derived from a midpregnant mouse mammary gland and capable of hormone-dependent differentiation in vitro. This cell line was cloned from the mouse Comma-D1 cell line (see below).
- Comma-D1 mouse non-tumorigenic, non-metastatic mammary epithelial cells derived from a midpregnant mouse mammary gland and capable of hormone-dependent differentiation in vitro.
- NIH/3T3 Mouse Fibroblasts
- Primary mouse mammary epithelial cells
- Primary mouse fibroblasts
- Murine fibroblasts (3T3-L1), a substrain of NIH/3T3 fibroblasts which undergo a pre-adipose to adipose-like conversion.
- Murine Adipocytes (Differentiated from 3T3-L1)
- Primary human fibroblasts

Characteristics of these cell lines, along with others previously mentioned, are listed in Table 1.

Furthermore, MDA-231GFP variant CM was obtained. As stated in Aim 2 (see below), MDA-MB-231PY, MDA-MB-231BO, and two other added human cell lines, MDA-MB-231W and MDA-MB-231BRMS human cancer cells, were injected into athymic nude mice via intracardiac inoculation. CM from these cells were obtained and assayed for cytokine expression (Task 1b) to ensure MDA-231-GFP variant counterparts

were expressing 1) the same cytokines in 2) similar quantities as their non-GFP counterparts.

Finally, culture supernatant from cell lines used for intracardiac inoculations (MDA-MB-231W-GFP, MDA-MB-231PY-GFP, MDA-MB-231BO-GFP), as well as MDA-MB-231W parental cells were all tested for mycoplasma to ensure cell purity and rule out the possibility of cell contamination contributing to cytokine expression. Cells were grown in antibiotic-free media for at least three passages. All cell lines tested negative for mycoplasma, as seen in Figure 1. The assay that was used for this test, the PCR Mycoplasma Detection Set from TaKaRa Bio, Inc., can detect mycoplasma levels as low as 10 pg-1 ng for the first PCR reaction (detection limits are dependent on the species of mycoplama present) and 10 fg-100 fg for the second PCR reaction. Cell lines to be assayed in the coming weeks include remaining major cells MDA-MB-231BRMS-GFP, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, MDA-MB-468P, and hTERT-HME1.

Task 1b: Quantitate selected inflammatory cytokines (IL-6, IL-8 [MIP-2], MCP-1, and KC) using standard ELISAs. Species-specific ELISAs and Bio-Rad Bio-Plexes™ were used to quantitate the amount of inflammatory cytokines present in the collected CM. Human cytokines examined for (6: IL-6, IL-8, GRO-alpha, MCP-1, VEGF, and SDF-1) were narrowed down from a larger group (32) as listed in the previous grant report. Select CM, relevant to future experimentation, was tested for cytokine content due to the expense. CM assayed for cytokine content included MDA-MB-231W, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, hTERT-HME1, MDA-MB-468P CM, and MDA-MB-231-GFP variant counterparts MDA-MB-231W-GFP, MDA-MB-231PY-GFP, MDA-MB-231BO-GFP, and MDA-MB-231BRMS-GFP CM. MCF-10A, HBL-100, HC-11, Comma-D1, primary human fibroblasts, primary mammary epithelial, primary mammary fibroblasts, NIH/3T3 fibroblasts, 3T3-L1 fibroblasts, and murine adipocytes were NOT tested for their CM cytokine expression as these cell lines were not used for experimentation beyond task 1c-d. Additional CM batches of MDA-MB-231W, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, and hTERT-HME1 were prepared and tested for their cytokine content. Four different batches of CM from each of the listed cell lines were tested to assess variation in cytokine concentrations between batches. Values, as shown in Figure 2a-g, have NOT yet been corrected for cell number or DNA content. Experiments to determine the DNA content of the various batches of CM is currently underway. DNA content will be used to normalize the cytokine concentrations in CM based on cell number.

As was stated in the previous grant report, human MCP-1 is notably absent or in small quantities in each of the human CM batches examined. This is especially true for the MDA-231 variants tested. To ensure that this was indeed a true result and not a complication with the Bio-plex assay, CM from the 7 main cell lines used for testing (MDA-MB-231W, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, hTERT-HME1, and MDA-MB-468P) were additionally tested via a MCP-1 standard human ELISA to confirm results. As can been seen in Figure 3a-b, results between the two methods, Bio-Rad Bio-plex and standard human ELISA, are very similar. Thus, human MCP-1 is produced in very little or no amounts in the 7 main cell

lines used for experimentation. This is especially true for the human metastatic MDA-MB-231 breast cancer variant CM.

Furthermore, MDA-231GFP variant CM was obtained. As stated in Aim 2, MDA-MB-231PY-GFP, MDA-MB-231BO-GFP, in addition to MDA-MB-231W-GFP, and MDA-MB-231BRMS-GFP cells were injected separately into athymic nude mice via intracardiac inoculation. Cytokine concentrations were measured in MDA-231-GFP variant CM to ensure concentrations were similar to their non-GFP counterparts. As can be seen in Figure 4, cytokine concentrations found in the CM of MDA-231-GFP variants were comparable to their non-GFP counterparts. Again, notably absent or present in small quantities is human MCP-1.

In addition to the improvements stated in the previous annual report, several more upgrades to this task have been carried out: i) the addition of various cell lines used in task 1c-d to examine for differences in osteoblast-derived cytokine production that may be attributed to cell species, cell tumorigenicity or metastatic potential, or cell origin. As was described in the previous grant report, an unexpectedly large increase in osteoblast-derived cytokine production was seen when osteoblasts were treated with non-metastatic (hTERT-HME1 and MDA-231BRMS) cell CM. Thus, these additional cell lines (outlined in table 1 and above) were added to experiments to further define the osteoblast-derived cytokine response. Furthermore, ii) MDA-MB-231-GFP variant CM was obtained and assayed for cytokine expression. This was to ensure cytokine expression was comparable to their non-GFP counterparts. Since these GFP variants were to be used for intracardiac inoculation in mice, we wanted to be sure that cytokine concentrations obtained from that set of experiments (Aim 2) could be compared to in-vitro results (in-vitro vs. in-vivo). Finally, iii) major cell lines used for experimentation (7: MDA-MB-231W, and GFP variants used for intracardiac inoculation in Aim 2, MDA-MB-231W-GFP, MDA-MB-231PY-GFP, and MDA-MB-231BO-GFP, were assayed for the presence of mycoplasma to ensure for cell purity and rule out possible alterations in cytokine expression due to cell contamination. All cells tested negative for mycoplasma presence at the femtogram level. The remaining cell lines, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, MDA-MB-468P, hTERT-HME1, and MDA-MB-231BRMS-GFP, will be tested in the coming weeks.

Task 1c-d: Collect culture media from MC3T3-E1 osteoblasts at different stages of differentiation (task 1a) cultured for 24 hours with conditioned medium (CM) at 10, 25, or 50% of the cells listed in task 1a. Perform a RayBio® mouse antibody array. D. Quantitate selected inflammatory cytokines (IL-6, MIP-2 [IL-8], MCP-1, and KC) using standard ELISAs. MC3T3-E1 osteoblasts were grown to either 10, 20, or 26 days. MC3T3-E1 osteoblasts grown to 4 days were eliminated from further study as the osteoblast response to CM was low at this time in comparison with osteoblasts grown to days 10 or 20. Overall trends seen at the 4 day time point were comparable to those seen at the 10 and 20 day times. Osteoblasts were previously stained for alkaline phosphatase expression (bone turnover) and von Kossa (mineralization) to verify their stage of differentiation as stated in the previous report. Differentiation media was replaced with either 0 or 50% CM from the cells listed in Task 1a. Twenty-four hours later, culture media was collected, centrifuged to remove any debris, and stored at >-20°C until use. This experiment was conducted in at least duplicate and repeated twice for a total of $n \geq 4$

samples collected per condition. Murine ELISAs were used to determine if osteoblast-derived cytokine response to the addition of various non-metastatic CM differed based on cell species, tumorigenicity or metastatic potential, or cell origin. Murine IL-6 and MCP-1 were used as representative cytokines. It was found that osteoblast-derived cytokine expression was elevated with treatment of non-metastatic CM. This was independent of cell species (mouse vs. human), cell origin (epithelial vs. fibroblast/mesenchymal), or tumorigenicity. Surprisingly, in most cases, the osteoblast-derived cytokine expression of osteoblasts treated with non-metastatic CM was above and beyond the osteoblast-derived cytokine expression of osteoblasts treated with human metastatic CM. This result can be seen in Figure 5a-b, where 10 and 26 day old osteoblasts treated with MCF-10A CM expressed larger amounts of murine IL-6 and MCP-1 than when treated with MDA-231W, MDA-231BRMS, or two other human breast cancer cell variants, MDA-MB-435 or MDA-MB-435BRMS CM. Figure 6 further illustrates this point, with murine osteoblast-derived IL-6 and MCP-1 levels well above what was seen with human metastatic CM treatment.

Next, MC3T3-E1 osteoblasts were grown to 10 days and treated with CM collected from MC3T3-E1 osteoblasts grown to either 10 or 20 days. This experiment was conducted to determine if the observed osteoblast-derived cytokine response was treatment-type specific or simply due to the act of the treatment itself. As can be seen in Figure 7, there are no significant changes in the osteoblast-derived cytokine response when osteoblasts are treated with their own CM (note the scale bar). Osteoblast treatments include CM derived from osteoblasts grown to 4, 10, or 20 days, with CM collected after 24 hours, and CM derived from osteoblasts grown to 10 or 20 days with CM collected after 12 hours. Thus, the osteoblast-derived cytokine response that is seen is indeed CM treatment type specific.

Next, Bio-Rad Bio-Plex™ 5 x-Plex Murine Cytokine Assays were used to further quantitate the MC3T3-E1 osteoblast-derived cytokine response to 50% CM of the 7 main cell lines tested (MDA-MB-231W, MDA-MB-231PY, MDA-MB-231BO, MDA-MB-231BR, MDA-MB-231BRMS, hTERT-HME1, and MDA-MB-468P). Additional experimentation was carried out to obtain a larger n value for statistical significance. The 5 murine cytokines assayed for include murine IL-6, KC, MCP-1, VEGF, and MIP-2. These cytokines were narrowed down from a larger array (32) previous conducted. The original Bio-Rad Bio-Plex™ 32 x-Plex Murine Cytokine Assay, as listed in the previous grant report, consisted of the following cytokines:

- IL-1α
- IL-1β
- IL-2
- IL-3
- IL-4
- IL-5
- IL-6
- IL-9
- IL-10
- IL-12 p40
- IL-12 p70
- IL-13
- IL-17
- Eotaxin
- G-CSF
- GM-CSF
- IFN-γ
- KC
- MCP-1
- MIP-1α
- MIP-1β
- RANTES
- TNF-α
- IL-15
- IL-18
- FGF-basic
- LIF
- M-CSF
- MIG
- MIP-2
- PDGF-BB
- VEGF

The osteoblast response to CM from metastatic and non-metastatic cells was then assessed.

Figure 8 and Figure 9 show 4 graphs each. Graphs a and b are experiments conducted when MC3T3-E1 osteoblasts were grown to 10 days and graphs c and d represent experiments where MC3T3-E1 osteoblasts were grown to 20 days. Graphs a and c represent osteoblasts treated with batch 1 of CM and graphs b and d represent osteoblasts that were treated with batch 2 of CM. The experiment utilizing batch 1 CM was conducted at time X and the experiment utilizing batch 2 CM was conducted at time Y. Although the results listed are biological replicates, there was variation among the replicates. Thus, the graphs were not combined due to high standard error. As can be seen in Figure 8 & 9a-d, there is a clear increase in osteoblast-derived cytokine production when osteoblasts are treated with non-osteoblast CM. In addition, osteoblasts naturally produce large amounts of MCP-1 compared to other cytokines (VM). MC3T3-E1 osteoblast-derived MCP-1 production is in the ng/ml range, compared with low to non-existent pg/ml ranges of the non-osteoblast CM tested in task 1b. As was noted in the previous grant report, when osteoblasts were grown to 10 days, the largest response in osteoblast-derived cytokine secretion was seen with the addition of CM from a brain-seeking variant (Figure 8a-d, orange slashed bar). At 20 days old, however, this response was not seen. In fact, at 20 days, there was no discernable trend with the largest osteoblast-derived cytokine response with any particular treatment. This potentially suggests that osteoblast age (differentiation stage) may be an important factor in examining the cytokine response to breast cancer cell treatments. Thus, for any future experiments conducted, as well as the osteoblast response to non-metastatic cell CM listed above (pertaining to Figures 5-7), osteoblasts grown to 10 days old will be used as a representative system.

As was noted in the previous grant report and further illustrated here, quite surprisingly, there was a very large osteoblast-derived cytokine response to the addition of non-metastatic cell CM (Figure 5, 6, and 9). This effect was repeatedly seen. The osteoblast-derived cytokine response to non-metastatic cell CM was, for the most part, greater than that seen with the addition of metastatic CM (particulary evident in Figure 5a-b).

Task 1e-f: Collect culture media from MC3T3-E1 osteoblasts at different stages of differentiation (task 1a) co-cultured at 100:1, 50:1, or 10:1 (osteoblast to non-osteoblast) for 24 hours with cells listed in task 1a. Perform a RayBio® mouse antibody array. F. Quantitate selected inflammatory cytokines (IL-6, MIP-2 [IL-8], MCP-1, and KC) using standard ELISAs. We then wanted to examine both the osteoblast-derived and *non-osteoblast*-derived cytokine response when the two cell types were put into a "co-culture" system with each other. While CM treatments yielded interesting results, these results are limited in application as the CM is unresponsive to the cells it is treated with. A physiological model would be better represented by two cell types that could respond to each other. Therefore, MC3T3-E1 osteoblasts were grown to either 10 or 20 days in 24 well plates. On day 9 or 19, each of the 7 main cell lines utilized for experimentation were plated in the culture inserts (3 μm pore size as determined via experiments listed in

the previous annual report). Human cells were plated separately in the inserts at the following osteoblast:non-osteoblast densities: "10:1" (5×10^4 osteoblasts to 1,890 non-osteoblast cells), "1:1" (5×10^4 osteoblasts to 18,900 non-osteoblast cells), and "1:2" (5×10^4 osteoblasts to 37,800 non-osteoblast cells). These cell numbers were determined based on culture dish and insert surface area (manufacturer recommended 1.89×10^4 cells / 33 mm^2 for a 24 well insert). Human cells were allowed to adhere overnight. Twenty-four hours later, all media were removed from wells and inserts, cells were rinsed with PBS, and media replaced with fresh differentiation media. 0.75 ml media was placed in the lower chamber and 0.3 ml media was placed in the upper chamber. Breast cancer cell inserts were transferred to wells containing 10 or 20 day old osteoblasts. The cell culture system was incubated for an additional three days at which point culture media was collected, centrifuged to remove any debris, and stored at >-20°C until use. For hTERT-HME1 and MDA-MB-468P cells, cells were plated in inserts 3 days earlier due to their slow and irregular growth compared to other cells used. This extended period was required to allow them to grow to the same cell numbers as the MDA-MB-231BRMS. In addition, MDA-MB-468P cells were plated at double the concentration due to their slow growth: "10:1" (5×10^4 osteoblasts : 0.378×10^4 MDA-468P cells), "1:1" (5×10^4 osteoblasts : 3.78×10^4 MDA-468P cells), and "1:2" (5×10^4 osteoblasts : 7.56×10^4 MDA-468P cells). This experiment was conducted in duplicate and repeated at least one additional time ($n \geq 4$ samples per condition). Figure 10 shows an illustration of the experimental set-up.

Figures 11 and 12 show the MC3T3-E1 osteoblast-derived cytokine response to human metastatic breast cancer cells (Figure 11) and human non-metastatic cells (Figure 12). As can be seen, while there is a slight increase in osteoblast-derived cytokine expression with the treatment of 10:1 cells (more osteoblasts to non-osteoblasts), this response is very small in comparison to the response seen with CM treatments. Thus, we chose to focus our attention on the "1:1" (same number of osteoblasts : non-osteoblasts) or "1:2" (less osteoblasts : non-osteoblasts) ratios. Comparison of these ratios is important, however, and does demonstrate a dose-response in cytokine expression (as described below in Figure 18) by both osteoblasts and non-osteoblast cells. Furthermore, Figures 13 and 14 show the human cell response to MC3T3-E1 osteoblast treatment at the 10:1 ratio. Figure 13 illustrates the metastatic cancer cell response, and Figure 14 illustrates the non-metastatic cell response. Several noteworthy points can be taken from these figures. First, most notably, human MCP-1 is again non-existent or produced in very small amounts by the human cells (metastatic or non-metastatic) when they are in a "co-culture" system with the murine osteoblasts. Once again, this directs attention to MCP-1 as being a cytokine of extreme interest in the inflammatory response that surrounds a foreign cell type. Second, osteoblast treatment with non-metastatic cells yields the production of larger amounts of human-derived cytokines than metastatic cells. This was a reproducible response. Finally, treatment with no cell type, metastatic or non-metastatic, stands out as being the "smallest or largest producer" of human derived cytokines. Thus, there is no differentiation amongst the metastatic cell variant treatments.

Focusing our attention on the "1:1" ratio of cells (same number of osteoblasts per mm^2 to : non-osteoblasts), in addition to treating the MC3T3-E1 osteoblasts with human metastatic or non-metastatic cell types, osteoblasts were also put into a "co-culture"

system with themselves (OB+OB). This was used as a control to ensure osteoblast-derived cytokine expression was treatment-type specific. Figures 15 and 16 show the MC3T3-E1 osteoblast-derived cytokine response to human metastatic breast cancer cells (Figure 15) and human non-metastatic cells (Figure 16). Due to high standard error between experiments, results were not averaged, but were plotted as individual experiments. As is illustrated at day 10, there is no strong increase in osteoblast-derived cytokine production when osteoblasts were in "co-culture" with human metastatic breast cancer cells. In fact, osteoblast-derived VEGF is decreased when compared with control (VM) and OB+OB. Very little or no MIP-2 was produced during treatment. What is of significant interest, however, is the large amount of osteoblast-derived MCP-1 that is expressed when compared with the other cytokines. MCP-1 is naturally produced in large amounts by the osteoblasts (VM), and is additionally produced in large amounts when osteoblasts were treated with their own cells (OB+OB). Osteoblast-derived MCP-1 expression does not decrease when treated with human metastatic breast cancer cells. In fact, when treated particularly with MDA-231W metastatic cancer cells, osteoblast-derived MCP-1 increases compared with control (VM). This can be seen throughout both experiments conducted (A & B). At day 20, trends comparatively change, once again suggesting the importance of osteoblast maturation stage to foreign cell response. As seen in Figure 15c, both osteoblast-derived IL-6 and VEGF are greatly increased above control (VM) with treatment of nearly all metastatic variants. Once again, of extreme interest is osteoblast-derived MCP-1, which, in as seen in both Figure 15c and d (reproduced result in two separate experiments), has significantly decreased with all metastatic cell treatments compared with control. This response was not seen at Day 10.

Osteoblast treatment with non-metastatic cell variants yielded somewhat different results. As can be seen in Figure 16a-b (osteoblasts grown to 10 days old), there was no dramatic increase or decrease in osteoblast-derived cytokine production with either MDA-231BRMS or MDA-468P cells. Osteoblast-derived cytokine production was significantly increased, however, with treatment of hTERT-HME1 mammary epithelial cells. This was again above and beyond osteoblast-derived cytokine response with treatment of metastatic breast cancer cell variants. At day 20, a slightly different cytokine expression pattern was seen. Osteoblast-derived cytokine response to treatment with hTERT-HME1 cells remained high in comparison to treatment with MDA-231BRMS and MDA-468P cells. However, when osteoblasts were treated with MDA-231BRMS and MDA-468P cells, osteoblast-derived MCP-1 and VEGF decreased in concentration when compared to control (VM). For osteoblast-derived MCP-1 expression, this pattern seems to be unique to osteoblasts grown to 20 days (late differentiation). Figure 17 illustrates the murine MC3T3-E1 cytokine response to treatment with "10:1", "1:1", and "1:2" MDA-MB-231W cells. It can be seen that altering the amounts of metastatic cancer cells, at least in these concentrations, plays a limited role in affecting MC3T3-E1 osteoblast-derived cytokine response. What is clearly seen, however, are trends as mentioned above. At day 10 (Figure 17a), osteoblast-derived VEGF expression is decreased with the addition of all ratios of MDA-231W cells, however osteoblast-derived MCP-1 expression is increased. These trends change by 20 days, where now osteoblast-derived IL-6 expression is largely increased over control (VM) with all amounts of cancer cells; osteoblast-derived VEGF expression precipitously increases with increasing amounts of cancer cell addition; and osteoblast-

derived MCP-1 expression is decreased with all ratios of cancer cell addition. Overall, these results suggest the importance of osteoblast stage of maturity when examining cytokine response to foreign cell types.

While the osteoblast-derived cytokine response to treatment with human metastatic and non-metastatic cell variants was not as dramatic as CM treatments, what was extremely interesting was a serendipitous result explored in further detail. Figure 18 illustrates the *non-osteoblast*-derived cytokine response to treatment with MC3T3-E1 osteoblasts (looking at the system from the other way around). To begin, as expected, there was a dose-response in non-osteoblast-derived cytokine expression, when non-osteoblasts were plated in the transwell system by themselves (no osteoblasts) for all cell variants assayed. These results would be comparable to that seen with CM human cytokine concentrations. In Figure 18a-l, compare the "1:1" ratio of cytokine expression with the "1:2". A greater amount of cytokine response is seen with treatment of a "1:2" ratio (more non-osteoblasts than osteoblasts) than what is seen with treatment of a "1:1" ratio (the same amount per mm^2 of non-osteoblasts : osteoblasts). Recall that no osteoblasts are in the system for this particular experiment. Once again, due to high standard deviation between experiments, results were graphed based on individual experiments. Trends are consistent throughout (compare A with B, C with D, etc.). Figure 18a-b represent human, non-osteoblast IL-6 response, c-d represent human, non-osteoblast IL-8 response, e-f represent, if present, human, non-osteoblast MCP-1 response, g-h represent human, non-osteoblast VEGF response, i-j represent human, non-osteoblast GRO-alpha response, k-l represent, if present, human, non-osteoblast SDF-1 alpha response. It is important to note the very small, if at all, amounts of human MCP-1 produced by the non-osteoblast cell variants (note the scale bar). This trend is consistent with results seen in other experiments as detailed above. Of extreme interest, however, is the non-osteoblast-derived cytokine expression of non-osetoblasts by themselves compared to the non-osteoblast-derived cytokine response with MC3T3-E1 osteoblasts in the "co-culture" system. Figure 19a-b2 illustrates these data. Once again, experiments were graphed individually due to high standard error amongst them (compare A with B, C with D, and so on). Regardless of osteoblast age (10 or 20 days old), the human, non-osteoblast derived cytokine expression drops, in many cases by more than half the original amount, when non-osteoblasts are placed in a "co-culture" system with osteoblasts. This was consistent for all non-osteoblast cell variants, irregardless of type (metastatic vs. non-metastatic). It can also be seen that MCP-1 is again expressed by the human, non-osteoblast cells in zero or very small concentrations. These results suggest that the non-osteoblast cells are highly responsive to the MC3T3-E1 osteoblasts. In fact, the non-osteoblast cells reduce their cytokine production precipitously when in a "co-culture" sytem with the MC3T3-E1 osteoblasts. It may be the case that the non-osteoblast cell variants are utilizing the cytokines produced by the MC3T3-E1 osteoblasts if needed, rather than producing their own. An alternative explanation could be that MC3T3-E1 osteoblasts express a factor that inhibits non-osteoblast-derived cytokine expression when the two cell types are in a "co-culture" system together.

Improvements to this task include examining the human, non-osteoblast-derived cytokine response, which turned out to be extremely important in our understanding of this system. As a control for this system, human, non-osteoblast cells were plated by themselves in a transwell system, and culture supernatants were obtained and assayed.

These were then compared with human, non-osteoblasts that were plated in a "co-culture" system with MC3T3-E1 osteoblasts. When examining the murine-derived cytokine response, a second "control" was added, which involved osteoblasts being treated with osteoblasts to ensure that the cytokine response was cell type-specific and not simply from being in a culture system with second cell type. No further experimentation is planned for task 1.

Task 2. **To determine how bone-derived inflammatory cytokine production is altered in response to breast cancer cells *in vivo*. (Months 13-17)**

Prior to any mouse work being done, two experiments were initially carried out using the cancer cell-GFP variants in order to ensure results obtained with them were comparable to results obtained with their non-GFP counterparts. As illustrated in Figure 21, MC3T3-E1 osteoblasts were grown to 10 days old and were treated with 50% CM obtained from human cancer cell-GFP variants. After 24 hours, culture supernatants were obtained and assayed for the presence of murine cytokines IL-6, KC, MCP-1, MIP-2, and VEGF using a Bio-rad Bio-plex murine cytokine quantification assay. As can be seen, all cancer cell-GFP variant CM caused an increase in osteoblast-derived cytokine expression over the VM control, similar to non-GFP counterparts. The MDA-231W-GFP variant CM, in particular, caused the largest osteoblast-derived cytokine response with all cytokines tested. Statistical analyses have yet to be run on this data, however it is expected that the MC3T3-E1 osteoblast-derived cytokine response to treatment with MDA-231W-GFP CM will be statistically significant at least at the $P \leq 0.05$ level for all cytokines tested compared with both VM and other cancer cell-variant CM. Furthermore, osteoblast-derived MIP-2 was found with treatment of the MDA-231W-GFP CM, whereas very little or zero osteoblast-derived MIP-2 was observed with treatment of the other cell variant CM. Second, CM from all MDA-231-GFP variants was assayed for the presence of human cytokine expression to compare with non-GFP counterparts as well as retrieved inoculated cells (see below for explanation). Figure 22a-c illustrates the human cytokine expression of all MDA-231-GFP variants when compared with retrieved cells (see explanation below). Human cytokine expression levels of MDA-231-GFP variants were comparable to their non-GFP counterparts (compare Figure 22a-c with Figure 2a, b, c, and e).

Figure 20 shows an illustration of the experimental set-up for murine intracardiac inoculations. 30 Harlan-Sprague-Dawley athymic, nude female mice were received on 11/14/07. They were born on either 10/8/07 or 10/19/07. Mice were kept quarantined for one week prior to any experimentation in accordance with university regulations. After the quarantine period was over, female athymic nude mice, 4-6 weeks in age, were inoculated in the left cardiac ventricle via intracardiac injection with 3×10^5 cells / 200 µl of MDA-231-GFP variants. Control mice were untreated. Notable changes to the original grant include the addition of two cell lines: MDA-MB-231W-GFP for comparison and MDA-MB-231BRMS-GFP as a non-metastatic control (in addition to MDA-MB-231PY-GFP and MDA-MB-231BO-GFP as originally proposed). We have extensive experience working with the MDA-MB-231W-GFP cell line in athymic nude mice. Another addition to the original grant included the use of Brefeldin A, a lactone antibiotic that interferes with cytokine secretion[9]. Brefeldin A blocks protein transport from the endoplasmic recticulum to the Golgi apparatus. Proteins will accumulate inside

the endoplasmic recticulum and thus remain inside the cell. Mice were split up into two groups, those that would be treated with Brefeldin A and those that would not. Both mice groups were allowed to live three weeks beyond injection. This time point was a modification of the original time points. In previous mouse studies, we have found that mice begin to die due to advanced tumor stage around the 4 week time point. Thus, maintaining mice to 4 or 8 weeks may be extremely difficult. In addition, in order to study the cytokine response to cancer cell metastasis, at the 1 and 2 week time points, we were unsure if sufficient metastases would have occurred to elicit a response detectable by our assays. Thus, we chose to utilize mice that had been euthanized at 3 weeks.

For the Brefeldin group, 6 hours prior to euthanization, mice were inoculated via tail vein injection with 500 µl of 0.5 mg/ml in PBS, further diluted from a stock concentration of 20 mg/ml Brefeldin A in DMSO as per Liu, et al[9]. This proved to be a rather difficult task for several reasons. 1) We were having significant problems with getting the brefeldin to go into solution at this particular concentration. With considerable manipulation, the brefeldin finally did go into solution (adding DMSO to the original PBS diluted solution), however it seems as though a 5 mg/ml concentration is the highest that can be made into 500 µl. 2) It proved extremely difficult to get the entire 500 µl of volume into our small mice. In many cases, we were only able to get half the volume in. Thus there was a logistical problem correctly getting the needed concentration of brefeldin in a small enough volume to inject into the mice. This experiment was attempted to be reproduced from Liu, et al[9]. All mice injected survived the 6 hours, although one began to look sickly near the 3 hour time point. From this point on, the mice were processed in the same manner: mice were euthanized at 3 weeks. To attempt to minimize an artificial increase in cytokine production with the use of CO_2 euthanasia, two mice were euthanized via cervical dislocation. Immediately following euthanization, and an addition to the original protocol, carcasses were immediately inverted, fine tweezers were utilized to remove an eye from the eye socket, and mouse blood was obtained via an eye bleed. The mouse carcass was gently massaged from tail to head in order to aid blood flow. What was quickly realized with the use of the two mice euthanized via cervical dislocation was that blood flow quickly ceases. Only a drop or two of blood was able to be collected utilizing this method. It may be the case that either 1) cervical dislocation induces a sympathetic nervous response to constrict blood vessels or 2) all heart function immediately stops causing little blood to be available from an eye bleed. We then decided to go back to the original protocol of CO_2 euthanization in order to facilitate collection of blood. Between 0.25 - 1 ml blood was easily collected using this method. Therefore, the rest of the mice were processed using CO_2 euthanization. For blood serum collection, whole blood was placed in a 4˚C refrigerator overnight and allowed to clot. The following day, blood was centrifuged at 1500 rpm for 3-5 minutes and the top serum fraction was collected. Blood serum was assayed for the production of murine cytokines using a Bio-rad Bio-plex murine cytokine array for serum samples. Additionally, murine femurs, and some tibias, fibulas, and full skeletons were harvested. All bones were initially placed into PBS and photographed using a light and fluorescent microscope to examine for the presence of tumors. Bones were then used for the following:

- Immunohistochemistry: whole bone femurs or tibias and fibulas were fixed in 4% paraformaldehyde for 24-48 hours at 4 degrees C. After bones were fixed, they were

then moved to a 0.5 mole/L EDTA solution at 4 degrees C for decalcification. They can remain in this 0.5 mole/L EDTA solution indefinitely and still retain fluorescence[10]. For use, bones were removed from the decalcification solution, snap frozen in liquid nitrogen via the CryoJane method, and sectioned into ~5-10 µm sections on a Leica Cryostat. Slides were stored at -20 degrees C for short term (~2-3 week) storage.

- Bone architecture: whole femurs and tibias were wrapped in saline soaked gauze and stored at -20 degrees C in 1.5 ml eppendorf vials. Bones were taken to a collaborator who can examine the bones via micro-CT for the presence of tumors.

- Bone cytokine analysis: Femur metaphyses were fractionated into ends (proximal to the hip and distal to the hip) and shaft. Bone marrow was flushed separately from bone sections with 1 ml PBS into a 1.5 ml eppendorf vial. Bone marrow from both ends were combined due to low volume. Isolated metasphyseal bone pieces were crushed and cultured separately in 1 ml alpha-MEM in separate wells of a 24-well plate. Crushed bones were incubated for 24 hours and culture supernatants were collected and tested after 24 hours for the presence of murine IL-6, MIP-2, KC, MCP-1, and VEGF using a Bio-Rad Bio-Plex™ murine cytokine quantification assay. Crushed bones were collected and frozen at -20 degrees C for potential future use.

- Flushed bone marrow: flushed bone marrow was initially centrifuged to collect bone marrow serum. The top serum fraction was collected and for murine cytokine expression of IL-6, MIP-2, KC, MCP-1, and VEGF using a Bio-Rad Bio-Plex™ murine cytokine quantification assay. The remaining cells were plated in a 24-well plate in it's respective cancer cell growth media (i.e. flushed bone marrow from a mouse inoculated with MDA-231BRMS-GFP cells was plated with MDA-231BRMS-GFP growth media). Any cancer cells present in the marrow were expanded, and retrieved cancer cells collected in order to obtain CM. CM was prepared by treating confluent cells with serum-free alpha-MEM for 24 hours. Retrieved cells were frozen for future use.

The many improvements to this aim include conducting immunohistochemistry on bone sections, assaying for bone architecture changes via micro-CT, and retrieving injected cancer cells for further analyses.

Overall, the breakdown of mice was as follows:

- Control: 4 (Brefeldin 2; 150-350 µl)
- MDA-231-GFP (W): 5 (Brefeldin 2; 50 µl)
- MDA-231PY-GFP: 6 (Brefeldin 2; 480-500 µl)
- MDA-231BO-GFP: 6 (Brefeldin 2: 150-250 µl)
- MDA-231BRMS-GFP: 5 (Brefeldin 2: 50-450 µl)
- Died in treatment: 1 accidental lung puncture (BRMS group), 1 -231GFP (W) after 24 hrs, 2 -231BO-GFP after ~30-60 min. from potential blood / cancer cell clot.

Two initial observations became apparent. First, nearing the three week time point, two mice from the MDA-231PY-GFP group began to look very sickly and quickly lost weight. They were both euthanized and processed one day prior to the others. Second, in the mice that were injected with the Brefeldin A, regardless of the volume of Brefeldin that they received, their bones were of a different color than those mice not injected with Brefeldin. As can be seen in Figure 22, the mice injected with Brefeldin had bones that were a "fleshy" red colored after decalcification, compared with greenish-grey colored

mouse bones from mice not injected with Brefeldin. Thus, it seems as though a chemical reaction did take place in the mice injected with Brefeldin, regardless of the volume.

When fluorescent pictures were taken of the bones, it quickly became apparent that whatever tumors were present were very small in size. As can be seen in Figure 23, five femurs (at least one from each experimental group) were identified as being positive for tumor presence via fluorescence microscopy. Tibias corresponding to these femurs also were identified as positive for tumor presence via fluorescence microscopy (not pictured). Cancer cells were found to traffic both to the distal (MDA-231W-GFP cells; Figure 23a) and proximal (MDA-231PY-GFP, MDA-231BO-GFP, and MDA-231BRMS-GFP cells; Figure 23b-d) ends. As found in a previous study[11], cancer cells predominantly traffic to the distal ends of long bones first, and later migrate to the proximal end followed by the shaft. The fluorescence intensity also varied considerably with the MDA-231W-GFP cells being the brightest (Figure 23a) followed by the remaining GFP variants. It was noticed that the MDA-231PY-GFP and MDA-231BO-GFP variants did not exhibit the same bright fluorescence as the MDA-231W-GFP or MDA-231BRMS-GFP prior to injection. With the observation that only a few visible large tumors were found via fluorescence microscopy at 3 weeks, the use of immunohistochemistry to assay for anti-GFP is needed in order to detect tumor presence on a cellular level.

First, retrieved cells were assayed for their human cytokine presence and compared with the human cytokine expression of their non-injected counterparts. As is illustrated in Figure 24, retrieved cells MDA-231W-GFP (A), MDA-231PY-GFP (B), and MDA-231BO-GFP (C) all expressed smaller quantities of human cytokines (red or checked bar) than their non-injected counterparts (black bar). MDA-231BRMS-GFP cells were retrieved, but could not be expanded to sufficient numbers for CM collection or freezing. The reduction in human cytokine expression in the retrieved cells occurred regardless of if the cells were retrieved from the femur ends or shaft. Thus, it seems as though the breast cancer cell variant cytokine production was altered in vivo in response to the murine bone microenvironment. The mechanism by which this occurred is unknown (i.e. was this an autocrine feedback mechanism, was this in response to a certain bone microenvironment factor, or was there a paracrine signal from the bone osteoblasts to decrease cytokine production?). Recall that when in a "co-culture" system with MC3T3-E1 osteoblasts, breast cancer cell cytokine production decreased, in all cases by at least half, compared to baseline values (Figure 19a-b2). In addition, human MCP-1 concentration remained extremely low or non-existent.

Second, murine femurs were fractionated into ends proximal to the hip, distal to the hip, and shaft, crushed and cultured for 24 hours, and culture supernatant assayed for murine cytokine production by a Bio-rad Bio-plex murine cytokine quantification assay. Figure 25 illustrates the murine cytokine concentration of femur metaphyses and diaphyses ex-vivo. As can be seen in Figure 25a-l, metaphyses (ends) of bone cleared of bone marrow in mice inoculated with MDA-231-GFP variants (colored and checked bars) produced substantially greater amounts of KC, MIP-2, IL-6, VEGF, and MCP-1 compared with non-cancer bearing control mice (black bars). This same phenomenon was seen in the bone diaphyses (shaft). Comparatively speaking, however, substantially larger amounts of cytokines were produced in the femur metaphyses (~1 - 10 ng/ml) than in the bone diaphyses (~0.02 – 3 ng/ml). Recall that the ends of the femur are to where metastatic breast cancer cells predominantly traffic[11]. Thus, these results implicate the

murine cytokines as important factors in the metastasis of breast cancer to bone. Although further statistical analyses are needed, there was no noticeable trend with murine cytokine expression between the MDA-231-GFP variants injected (i.e. cytokine expression in a MDA-231W-GFP inoculated mouse vs. MDA-231BO-GFP inoculated mouse). It was noticed, however, that there was a high degree of variation among the control mouse proximal to the hip bones in the two control mice assayed. These results can be seen in Figure 25m. Neither mouse appeared sickly, however it cannot be ruled out that one mouse had a low lying infection that may be affecting these results. Thus, as can be seen in graphs 25a, d, g, and j, the standard deviation for the control mouse is rather large. We are presently looking into a way to correct for this large deviation.

Third, the cytokine expression of murine femur metaphyseal bone marrow serum was assayed for murine cytokine production by a Bio-rad Bio-plex murine serum cytokine quantification assay. Bone marrow serum was assayed based on the ends (proximal to the hip and distal to the hip assayed together due to low volume) and shaft. As can be seen in Figure 26a-h, there was not much change in bone marrow serum murine cytokine expression of cancer-bearing mice (colored and checked bars) versus control mice (black bar). This could be due to low bone marrow volume (recall the bone marrow was obtained by flushing with 1 ml PBS). Flushing with 1 ml of volume may have inadvertently diluted the serum cytokines too low, although this effect would be seen across the bones assayed. In addition, serum samples were required to be diluted even further for the Bio-plex assay, thus cytokine levels may have been diluted below realistic detectable limits. Regardless, compared with murine cytokine expression of other experiments, the bone marrow cytokine expression was extremely low (~1-50 pg/ml compared with ng/ml levels of other experiments).

Finally, blood serum was assayed for the presence of murine cytokines using a Bio-rad Bio-plex murine serum cytokine assay. Figure 27a-c illustrates murine cytokine concentration of control mice (black bar) vs. A) MDA-231W-GFP, B) MDA-231PY-GFP, or C) MDA-231BO-GFP (solid red or black checked bar), and MDA-231BRMS-GFP (blue or orange checked bar) inoculated mice. While there was not much difference between the serum cytokines from the various MDA-231-GFP inoculated mice, a few notable observations can be made. First, murine blood serum MCP-1 is higher in both of the mice inoculated with MDA-231BRMS-GFP than the control. On average, the MCP-1 blood serum concentration of the mice inoculated with the MDA-231BRMS-GFP cells was higher than the MDA-231W-GFP cells, but was comparable to the blood serum obtained from mice inoculated with MDA-231PY-GFP and MDA-231BO-GFP cells. Also, on average, blood serum obtained from mice inoculated with MDA-231PY-GFP or MDA-231BO-GFP breast cancer cells yielded higher amounts of murine IL-6 than control or mice inoculated with MDA-231BRMS-GFP breast cancer cells. Very little change was seen in murine MIP-2 or VEGF cytokine expression of blood serum and KC blood serum cytokine concentrations were below detectable limits (2 pg/ml). Once again, blood serum samples were required to be diluted for the Bio-plex assay, thus cytokine levels may have been diluted below realistic detectable limits. Again, compared with murine cytokine expression of other experiments, the blood serum cytokine expression was extremely low (~1-70 pg/ml compared with ng/ml levels of other experiments).

Analyses for determining bone architecture and immunohistochemistry are currently underway. A colleague was able to derive a crude overlay of a control femur

showing the trabecular bone (Figure 28). We aim to use this technology to label the tumor location within some of our femurs. Further results will be available in the final grant report. Immunohistochemistry of the three week bone sections is proving to be extremely difficult. We chose to use cryosections instead of paraffin. Our main problem is that the bone sections do not stick well to any slides that we use (i.e. the section falls off when submerged in liquid). We have tried both charged and poly-L-lysine coated slides as recommended by our collaborators and pathologists. A colleague suggested using StarFrost adhesive coated slides that are specific for bone sections and fatty tissues. We are currently in the process of getting these slides and trying them for IHC analyses. Progress on this area of the grant will be noted in the final grant report.

Task 3. **To determine if osteoblasts and breast cancer cells have receptors and can respond to *osteoblast*-derived inflammatory cytokines. (Months 18-33)**

Significant updates to the literature have been made since the writing of this grant. These updates can be applied to several sections of task 3.

Task 3a: Based on the results of tasks 1 and 2, detect MC3T3-E1 osteoblast receptors to selected cytokines using fluorescently labeled receptor antibodies and analyze using flow cytometry. Literature has shown that MC3T3-E1 osteoblasts express receptors for and can respond to MCP-1 (CCR1/2[12]), IL-6 (IL-6R[13]), and VEGF (VEGFR1/Flt[14], and VEGFR3/NRP1[14]). It is unknown whether MC3T3-E1 osteoblasts express receptors to IL-8/MIP-2 (CXCR1/2), KC/GRO-alpha (CXCR1/2), or VEGFR2/KDR. Based on previous results as outlined above, we feel that this is no longer a viable question due to the present direction of the research. What is of more interest and of particular importance to the theme of this grant, however, is if cancer cells express receptors to these cytokines and can respond to chemotactic gradients. Part of this research question is outlined in Task 3e: Based on the results of tasks 1 and 2, detect MDA-MB-231P, MDA-MB-231BO, MDA-MB-231BR, and BRMS1 transfected-MDA-MB-231 cell receptors to selected cytokines using fluorescently labeled receptor antibodies and analyze using flow cytometry. Since the writing of this grant, literature has shown that MDA-231W cells in particular have receptors for and can respond to all of the major cytokines examined in this grant: IL-6 (IL-6R[15]), IL-8/MIP-2 (CXCR1/2[16]), GRO-alpha/KC (CXCR1/2[16]), MCP-1 (CCR1/2[16]), and VEGF (VEGFR1/Flt[17, 18] [also found on MDA-468P non-metastatic breast cancer cells), VEGFR2/KDR[18], and VEGFR3/NRP1[18, 19]). Based on previous results suggesting that there is minimal, if any, difference between metastatic breast cancer cell variants (MDA-231W vs. MDA-231PY, MDA-231BO, or MDA-231BR), we chose to use MDA-MB-231W human metastatic breast cancer cells as a representative human metastatic breast cancer cell line for future experiments. To that end, it is of most importance to the theme of this grant to determine if MDA-MB-231W metastatic breast cancer cells were chemoattracted to the major cytokines identified. It is expected that the MDA-231W cells will migrate towards gradients of IL-6, MCP-1, KC/GRO-alpha, VEGF, and/or IL-8/MIP-2 since they are known to express receptors for all ligands. This experimentation is proposed as task 3f: Based on the results of tasks 1 and 2, detect cytokines involved in breast cancer cell variant (task 3e) chemoattraction using a transwell system. Results for this task should also hold true and will be substituted for tasks 3g and h: Detect cytokines involved in breast cancer cell variant

(task 3e) invasion using Matrigel™. H. Detect cytokines involved in breast cancer cell variant (task 3e) migration using a wound healing assay. Estimate wound closure using light microscopy. Preliminary experiments for task 3f are currently underway. Using MDA-MB-231W human metastatic breast cancer cells as a representative metastastic breast cancer cell line and MDA-MB468P cells as a representative non-metastatic breast cancer cell line, we have shown that MDA-231W metastatic breast cancer cells migrate towards conditioned medium produced from both 10 day and 20 day old MC3T3-E1 osteoblasts after 24 hours (several hundred cells), whereas MDA-MB-468P cells migrate very little after 72 hours (~20 cells). Experiments are currently underway utilizing mouse IL-6, KC, and VEGF as chemoattractants at 1, 0.1, and 1 ng/ml concentrations respectively. We are also assaying for the effect of neutralizing these cytokines in the MC3T3-E1 osteoblast CM on MDA-MB-231W metastatic cancer cell migration. These experiments are all being conducted with BD™ Falcon FluoroBlok™ inserts with 8 µm pores. MDA-MB-231W cells are being stained with Vybrant DiI for visualization. Further details of these experiments will be available in the final grant report.

Literature has also shown that several of these cytokines can be growth and survival factors for MDA-MB-231 metastatic breast cancer cells, research which was to be carried out in task 3i: Detect cytokines involved in breast cancer cell variant (task 3e) proliferation using a proliferation assay (MTT Cell Proliferation). It has been shown that VEGF[17-19] (also with MDA-MB-468P non-metastatic breast cancer cells[17]), IL-6[15], and IL-8[20] all enhance MDA-231W metastatic breast cancer cell proliferation. It is unknown whether MCP-1 or GRO-alpha / KC have any effect on proliferation. It should be noted, however, that GRO-alpha binds to the same receptor as IL-8 (CXCR1/2). Thus, the likelihood of increased proliferation with GRO-alpha is high.

Task 3c and d: Collect culture media from RAW264.7 cells treated with MC3T3-E1 osteoblast culture media collected at 14 days and 20 days. Stain cells with TRAP stain. D. Count osteoclasts using a fluorescence light microscope. Literature has shown that, besides osteoblasts and metastatic breast cancer cells, osteoclasts also express receptors and can respond to cytokines identified through this research (IL-6 (IL-6R[21]), IL-8/MIP-2 (CXCR1/2[22]), GRO-alpha/KC (CXCR1/2[22]), MCP-1 (CCR1/2[23]), and VEGF (VEGFR1/Flt[24, 25], VEGFR2/KDR[25], and VEGFR3/NRP1[25]). Indeed, literature has also shown that several of these cytokines can induce osteoclastogenesis towards the production of a mature, functional osteoclast (IL-8[22], IL-6[26], VEGF[26], and MCP-1[23]). Working alongside the P.I. of this grant (Karen Bussard), an undergraduate researcher will be completing this aspect of the grant as part of his thesis work.

Task 3b: Examine for selected cytokine neutralization by ELISAs using TGF beta neutralizing antibody and PTHrP receptor antagonist (both cytokine mediators). PTHrP and TGF-β are reported to be present in breast cancer conditioned media and have been reported to induce IL-6, IL-8, or MCP-1[27, 28]. Thus, PTHrP and TGF-β were considered as possible factors secreted by MDA-231 metastatic breast cancer cells to promote osteoblast-derived cytokine expression. PTHrP was present in the breast cancer conditioned medium at 46 pg/ml (equivalent to ~2 x 10^{-12}M). TGF-β was present in the breast cancer conditioned medium at ~1-5 ng/ml. To test these cytokine mediators as potential promoters of osteoblast-derived cytokine expression, MC3T3-E1 osteoblasts

were grown to 16 days. Osteblasts were then treated for 24 hours with the following conditions: VM (nothing), MDA-231W CM, 2 ng/ml TGF- β_1 protein, 5 µg/ml anti-TGF- $\beta_{1,2,3}$ +/- CM, 2 ng/ml TGF- β_1 + 5 µg/ml anti-TGF- $\beta_{1,2,3}$, 5 x 10^{-9} M PTH (1-34) protein, 5 x 10^{-7} PTHrP (7-34) +/- CM, or 5 x 10^{-9} M PTH (1-34) protein + 5 x 10^{-7} PTHrP (7-34). In cases concerning a neutralizing antibody, CM was incubated with the antibody for 1 hour at 37 degrees C prior to addition to cells. Serum substitute was additionally used in experiments to rule out exogenous serum contributions of PTHrP or TGF- β. Culture supernatants were collected, centrifuged to remove debris, and assayed for the presence of murine IL-6 via standard ELISAs (IL-6 was selected as a representative cytokine). Results are illustrated in Figure 30. As is shown, IL-6 concentration is not reduced to VM levels by anti-TGF- $\beta_{1,2,3}$ or PTHrP (7-34) either alone or in combination. IL-6 concentrations are, however, reduced to levels comparable with CM treatment with the addition of both anti-TGF- $\beta_{1,2,3}$ or PTHrP (7-34). These data suggest that the combination of these factors is only partially responsible for reducing IL-6 levels in MC3T3-E1 osteoblasts. In light of these data, no further experiments are planned for task 3b.

Task 4. **Final data analysis and thesis preparation. (Months 34-36)**

Task 4 will begin shortly. Once all major experimentation is complete, statistical analyses will be performed for final result calculation. Final graph representation will be shown in the final grant report.

Progress in Program: From the time this proposal was submitted, all required course work for a Ph.D. has been completed. In December 2005, I passed my candidacy examination; and in June 2006, I successfully completed my comprehensive examination. I am currently finishing my 4th year of Ph.D. work.

KEY RESEARCH ACCOMPLISHMENTS

- Osteoblasts are an important source of KC, MCP-1, IL-6, and VEGF in the vicious cycle of breast cancer bone metastasis.
- Osteoblasts naturally produce large amounts of MCP-1.
- Breast cancer cell variants and their GFP counterparts do NOT produce MCP-1, but DO produce IL-8.
- The stage of osteoblast maturation is important in determining the osteoblast response to metastatic breast cancer cells.
- Osteoblast-derived cytokine expression is increased with treatment of CM from non-osteoblast cells. This effect is cell-type dependent (i.e. osteoblast-derived cytokine production did not increase when osteoblasts were treated with osteoblast CM).
- Osteoblast-derived cytokine production with treatment of non-metastatic cells or their CM is over and above that seen with treatment of metastatic breast cancer cell variants.
- The increase in osteoblast-derived cytokine production is independent of treatment cell species, origin, or tumorigenicity.
- Maximum induction of osteoblast-derived cytokine secretion at day 10 occurred with treatment of a brain-seeking variant. This effect was not seen at day 20.

- No difference among metastatic breast cancer cell variants was seen when osteoblasts and human cancer cell variants were placed in a "co-culture" system together.
- Osteoblast-derived MCP-1 decreased with treatment of MDA-MB-231 breast cancer cell variants and MDA-MB-468P non-metastatic cancer cells at day 20 in a "co-culture" transwell system.
- A limited dose response in osteoblast-derived cytokine production was seen with increasing amounts of non-osteoblasts in a "co-culture" transwell system.
- A distinct dose response was seen in human-derived cytokine production with increasing amounts of non-osteoblasts by themselves in a "co-culture" transwell system.
- Zero to low amounts of MCP-1 are produced by human non-osteoblast cells in a "co-culture" transwell system with murine osteoblasts.
- Human non-osteoblast-derived cytokine production decreased significantly when human non-osteoblasts were in a "co-culture" transwell system with murine osteoblasts.
- Brefeldin A induces a color change in murine bones.
- Retrieved human breast cancer cells had decreased cytokine production compared with non-injected human cancer cells.
- Zero to low amounts of MCP-1 are produced by human cancer cells ex-vivo.
- The metaphyses (ends) of bone in normal mice produce KC, MIP-2, IL-6, VEGF, and MCP-1.
- In cancer-bearing mice, the metaphyses (ends) of bone produce signficantly higher amounts of KC, MIP-2, IL-6, and VEGF than control or the bone diaphysis (shaft). Breast cancer cells predominantly traffic to the bone ends.
- No differences were seen amongst human cancer cell variants and their tumor formation or cytokine production in a murine model of breast cancer bone metastasis.
- Osteoblasts are an important source of cytokines, specifically MCP-1, in breast cancer bone metastasis.
- Cancer cell manipulation of the bone microenvironment facilitates tumor cell colonization and survival.

REPORTABLE OUTCOMES

Publications:

2008 Kinder, M, Chislock, EM, **Bussard, KM**, Shuman, LA, Mastro, AM. Metastatic Breast Cancer Induces an Osteoblast Inflammatory Response. Experimental Cell Research. 314: (1), 173.

2008 **Bussard, KM**, Gay, CV, Mastro, AM. The Microenvironment in Metastasis: What is Special About Bone? Accepted to Cancer Metastasis Reviews. Available on Epub ahead of print December 11, 2007.

2008 **Bussard, KM**, Mastro, AM. Osteoblast-derived MCP-1 Facilitates Bone Metastatic Breast Cancer. (working title) Manuscript in preparation.

Oral Presentations:

2008 **Bussard, KM**, Mastro, AM. "Osteoblast-Derived Cytokines are Major Mediators in Facilitating Bone Metastatic Breast Cancer." Presented at the American Association for Cancer Research Annual Meeting's Tumor Biology Minisymposium, April 12-16, 2008.

Abstracts / Poster Presentations:

2007 **Bussard, KM**, Mastro, AM. "Osteoblasts Naturally Produce Cytokines that Influence the Tumor Microenvironment in Bone Metastatic Breast Cancer." Skeletal Complications of Malignancy V, The Paget Foundation. October 25-27, 2007.

Research Opportunities Attended Based on this Research:

- American Association for Cancer Research – Edward A. Smuckler "Pathobiology of Cancer" Workshop selected attendee. Snowmass, CO: July 15-22, 2007.

Awards / Grants / Fellowships Won Based on this Research:

- American Association for Cancer Research – Scholar-In-Training Award: 2008
- The Pennsylvania State University College of Agricultural Sciences Travel Award: 2008.
- American Association for Cancer Research – Women in Cancer Research Brigid G. Leventhal Scholar Award in Cancer Research: 2006.
- Department of Defense Predoctoral Traineeship Award in Breast Cancer (Fellowship): 2006-2009.
- The Pennsylvania Space Grant Consortium NASA Space Grant Fellowship: 2005-2007, 2007-2009.

CONCLUSION

Overall, these data suggest that osteoblasts are an important source of cytokines, specifically MCP-1, in bone metastatic breast cancer. These data suggest that the following occurs: a) osteoblasts naturally secrete selected cytokines (IL-6, MCP-1, KC, MIP-2, and VEGF) at baseline levels. These may serve as chemoattractants for breast cancer cells (chemoattraction assays are currently underway) or may serve to prime the bone microenvironment for metastatic invasion. B) When a non-metastatic foreign cell enters the bone microenvironment, the osteoblast undergoes an inflammatory stress response and releases very large amounts of the same panel of selected cytokines (a "normal" inflammatory response). C) However, when metastatic breast cancer cells invade the bone microenvironment, osteoblast-derived cytokine production increases, but not to the extent seen with non-metastatic cell invasion. MCP-1, however, is secreted in significantly large amounts suggesting that this CC chemokine is a very important factor in breast cancer metastasis to bone. It is possible that cancer cell-derived cytokine production is muted during bone metastatic invasion to avoid eliciting an immune response. By circumventing any adverse inflammatory response, cancer cells are able to create a unique bone niche facilitating their growth and survival. In addition, literature

has shown that increased cytokine production of this cytokine panel leads to increased bone resorption via osteoclastogenesis, and the release of additional cancer cell maintenance factors such as TGF-β. Thus, these findings clearly implicate the bone microenvironment and cancer cell manipulation thereof in facilitating metastatic tumor cell colonization and survival.

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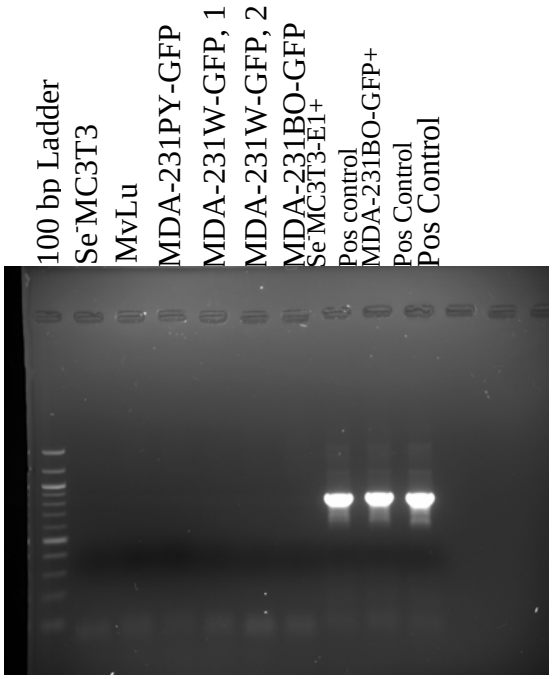
SUPPORTING DATA

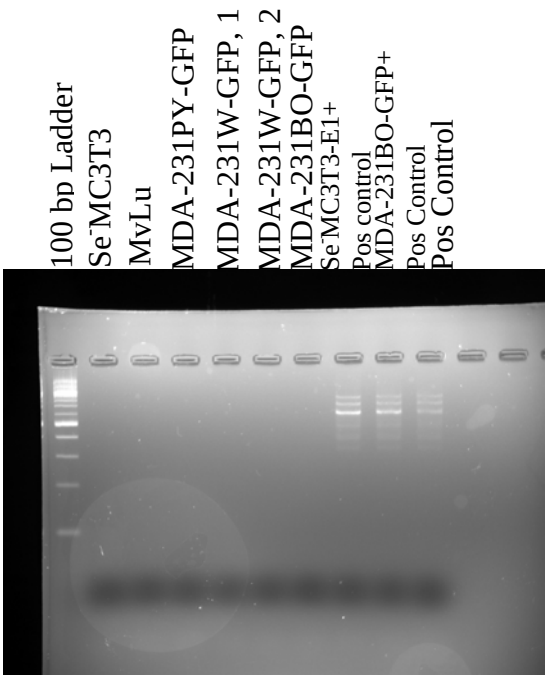
Table 1: Characteristics of Cell Types Tested

	Tumorigenic	Metastatic	Species	Type
MDA-MB-231	+	+	Human	Epithelial
MDA-MB-231PY	+	+	Human	Epithelial
MDA-MB-231BO	+	+	Human	Epithelial
MDA-MB-231BR	+	+	Human	Epithelial
MDA-MB-231BRMS	+	-	Human	Epithelial
hTERT-HME1	-	-	Human	Epithelial
MDA-MB-468P	+	-	Human	Epithelial
MCF-10A	+	-	Human	Epithelial
HBL-100	+	-	Human	Epithelial
Primary Human Epithelial	-	-	Human	Epithelial
HC-11	-	-	Murine	Epithelial
Comma-D1	-	-	Murine	Epithelial
NIH/3T3 Fibroblast	-	-	Murine	Fibroblast / Mesenchymal
3T3-L1 Fibroblast	-	-	Murine	Fibroblast / Mesenchymal
Adipocyte	-	-	Murine	Fibroblast / Mesenchymal
Primary Manmary Epithelial	-	-	Murine	Epithelial
Primary Mammary Fibroblast	-	-	Murine	Fibroblast / Mesenchymal

Figure 1: Mycoplasma Testing of Human Metastatic Breast Cancer Cell-GFP Variants Used in Intracardiac Inoculations.

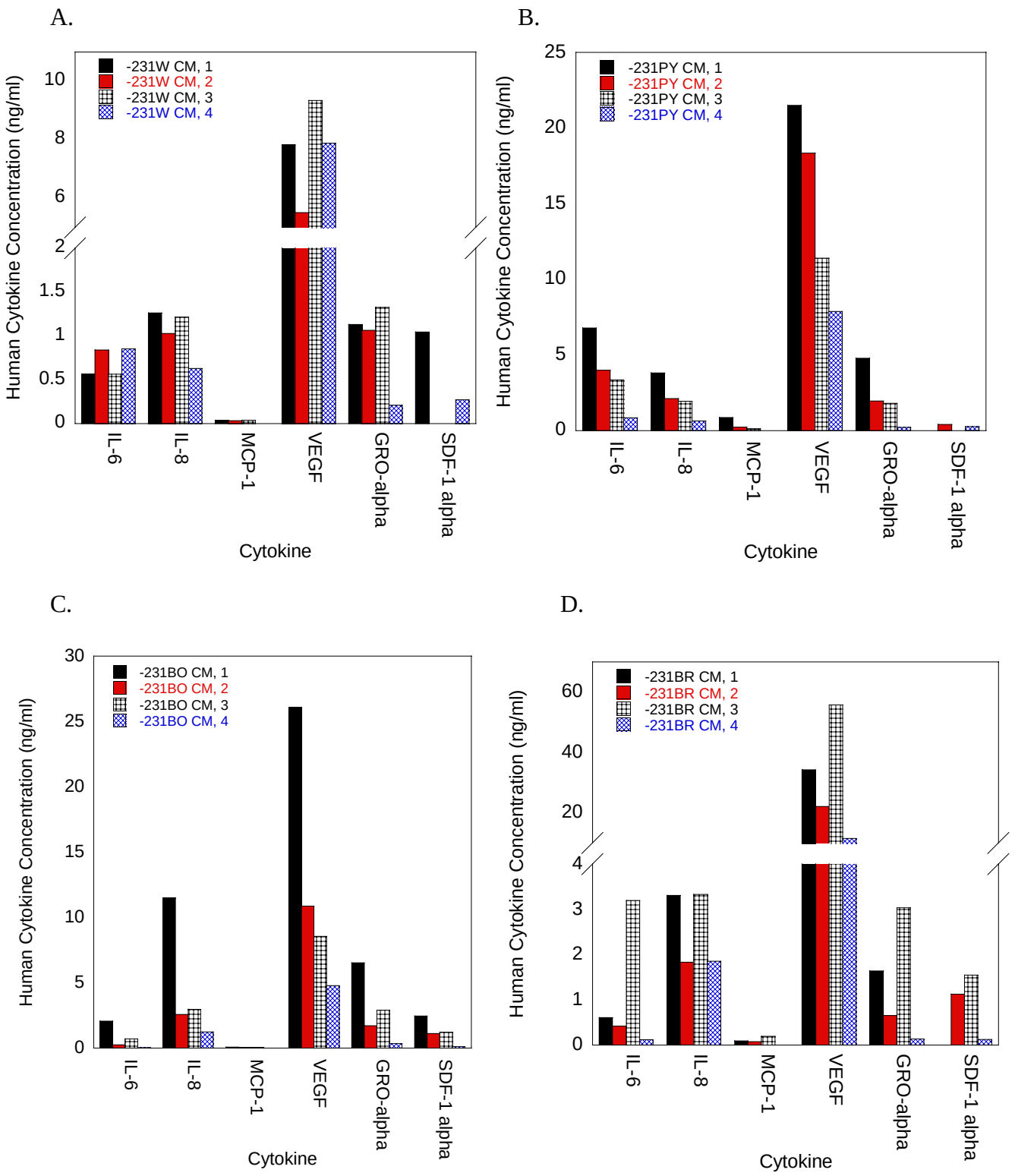


Reaction 1, 1% Gel



Reaction 2, 3% Gel

Figure 2: Cytokine Concentrations in various batches of MDA-231 human breast cancer cell variants.



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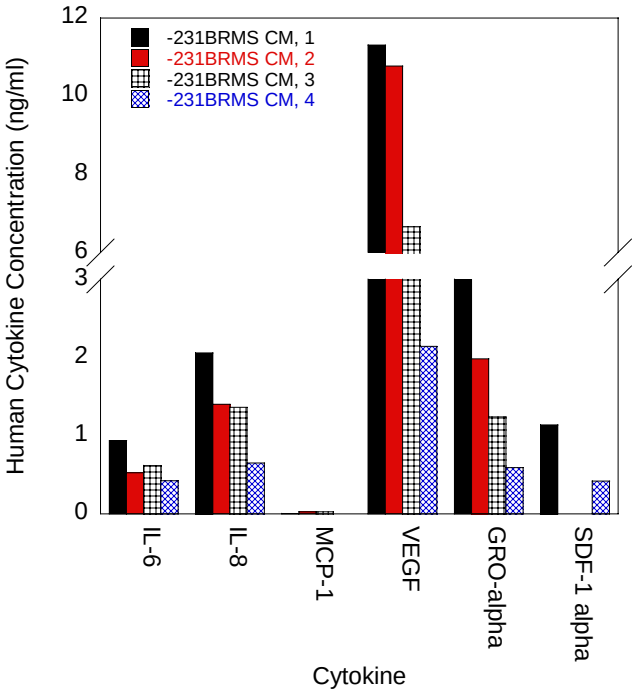
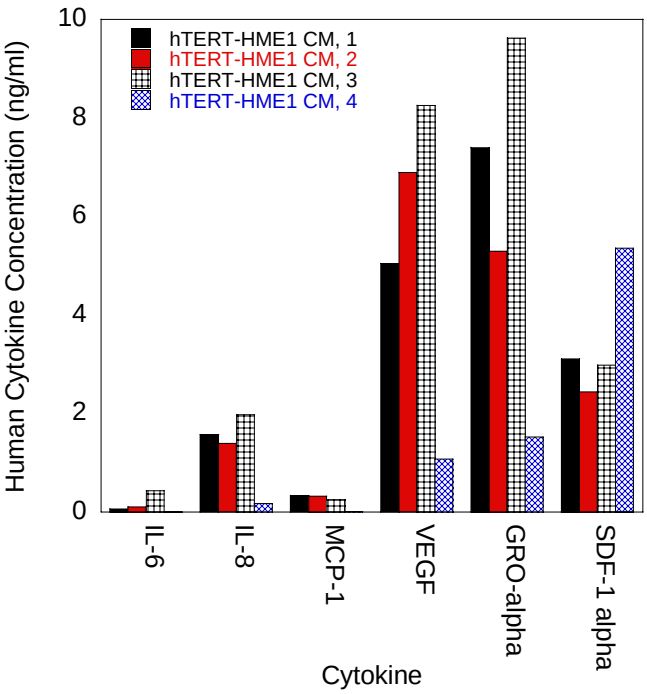
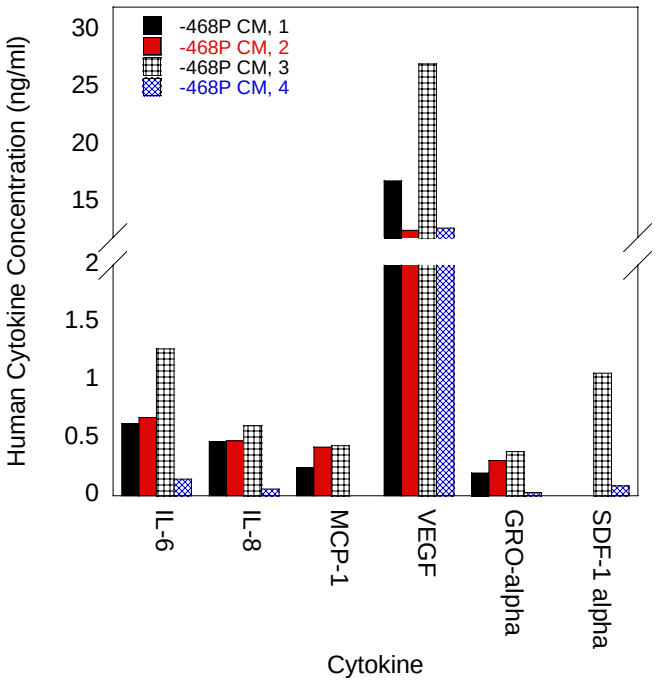

-231BRMS CM, 1
-231BRMS CM, 2
-231BRMS CM, 3
-231BRMS CM, 4
Human Cytokine Concentration (ng/ml)
12
10
8
6
3
2
1
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

F.


hTERT-HME1 CM, 1
hTERT-HME1 CM, 2
hTERT-HME1 CM, 3
hTERT-HME1 CM, 4
Human Cytokine Concentration (ng/ml)
10
8
6
4
2
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

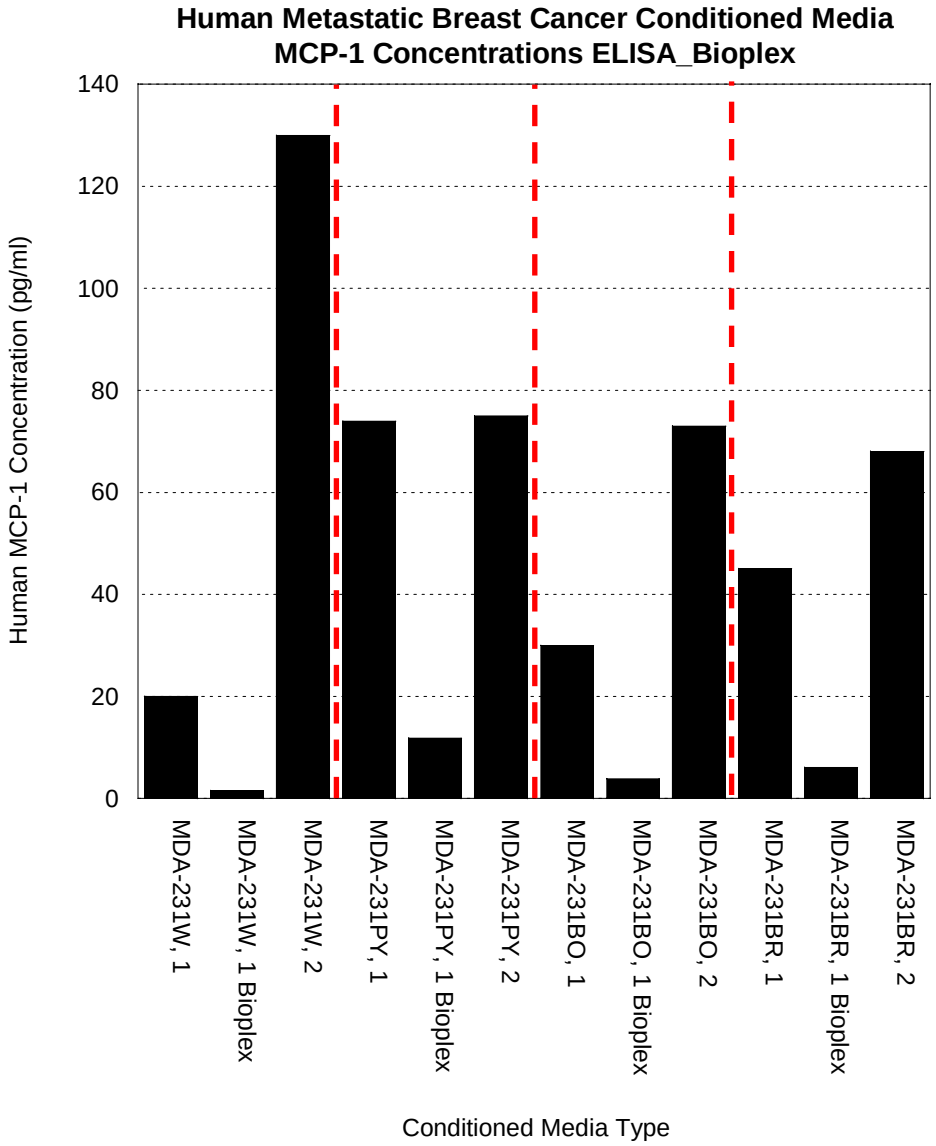
G.


-468P CM, 1
-468P CM, 2
-468P CM, 3
-468P CM, 4
Human Cytokine Concentration (ng/ml)
30
25
20
15
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

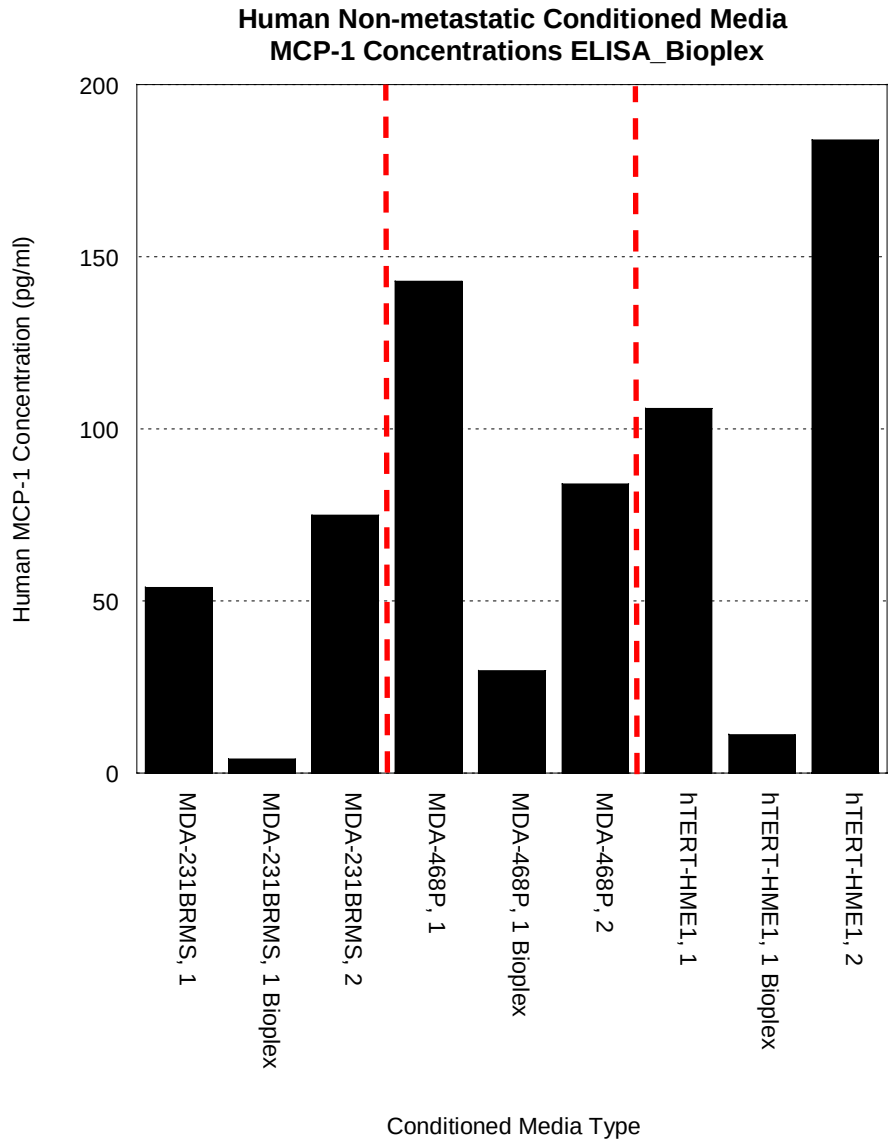
Conditioned media were prepared by growing cells to confluence. Growth medium was removed and cells were rinsed with PBS. Serum-free media (α-MEM, the base medium for MC3T3-E1 osteoblasts) was added to the breast cancer cells and was incubated for 24 hours. Conditioned medium was collected, centrifuged to remove any debris, and stored at >-20°C until used. Cytokines in the medium were quantified using human Bio-Rad BioPlex™ cytokine quantification assays.

Figure 3: Confirmation of MCP-1 cytokine concentrations in various batches of MDA-231 human breast cancer cell variants.

A.

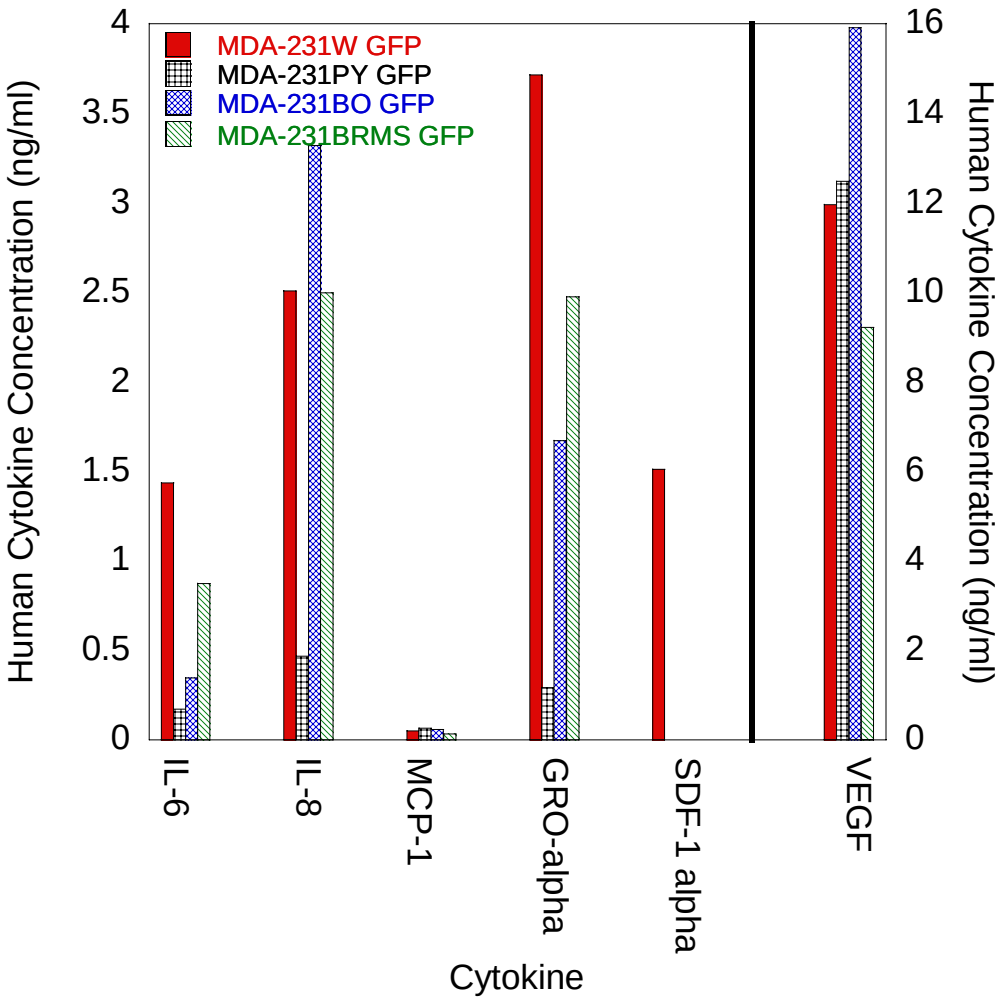


B.



Conditioned media were prepared by growing cells to confluence. Growth medium was removed and cells were rinsed with PBS. Serum-free media (α-MEM, the base medium for MC3T3-E1 osteoblasts) was added to the breast cancer cells and was incubated for 24 hours. Conditioned medium was collected, centrifuged to remove any debris, and stored at >-20°C until used. Cytokines in the medium were quantified using human Bio-Rad BioPlex™ cytokine quantification assays (batch 1, middle bar of grouping) and standard human ELISAs (batches 1 and 2, first and last bars of grouping).

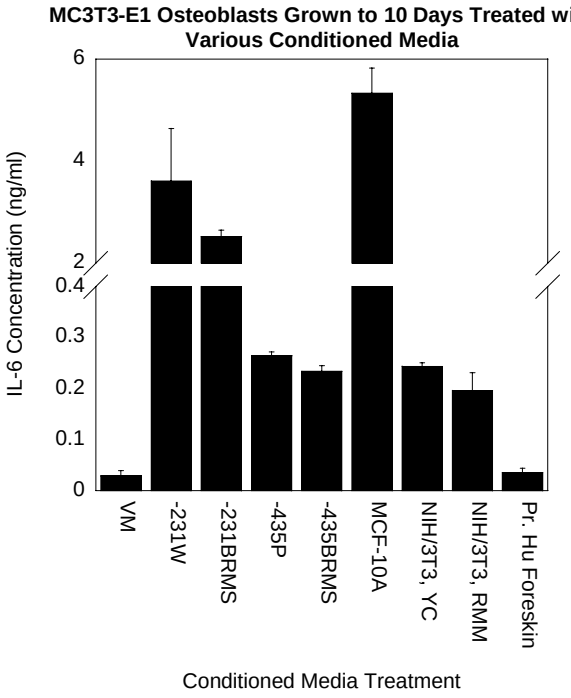
Figure 4: Cytokine concentrations in the conditioned media of MDA-231-GFP human breast cancer cell variants.

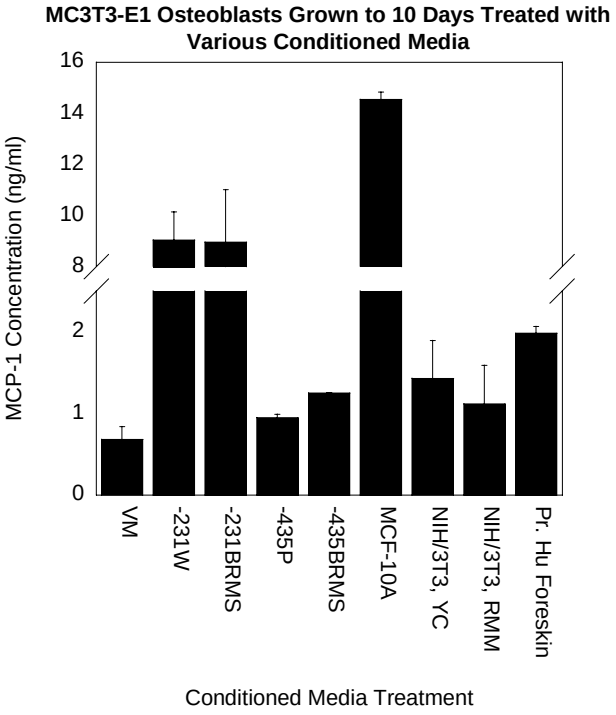


Conditioned media were prepared by growing cells to confluence. Growth medium was removed and cells were rinsed with PBS. Serum-free media (α-MEM, the base medium for MC3T3-E1 osteoblasts) was added to the breast cancer cells and was incubated for 24 hours. Conditioned medium was collected, centrifuged to remove any debris, and stored at >-20°C until used. Cytokines in the medium were quantified using human Bio-Rad BioPlex™ cytokine quantification assays.

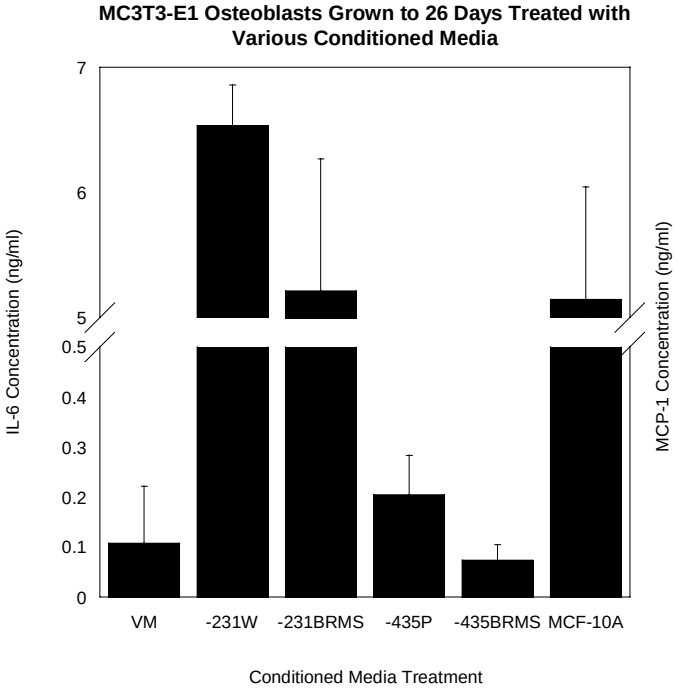
Figure 5.: Cytokine expression of MC3T3-E1 osteoblasts treated with 0 (VM) or 50% conditioned medium from various cell types.

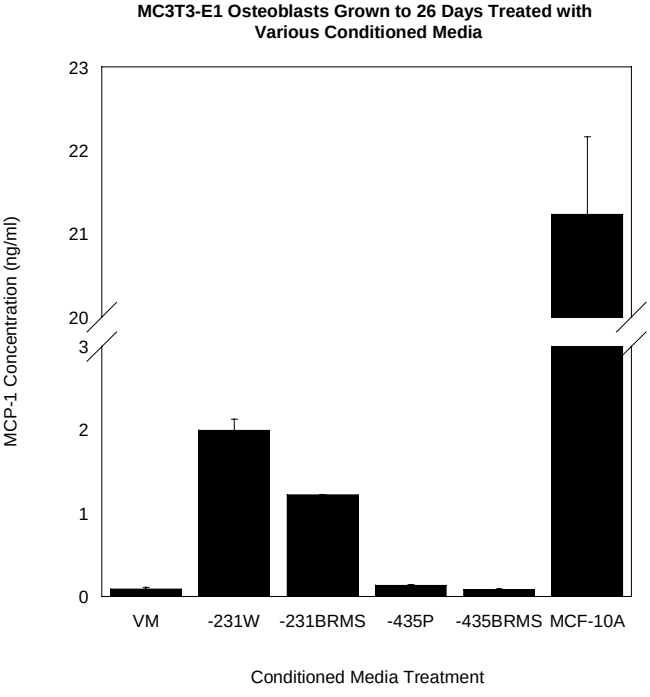
A. MC3T3-E1 Osteoblasts Grown to 10 Days Old





B. MC3T3-E1 Osteoblasts Grown to 26 Days Old

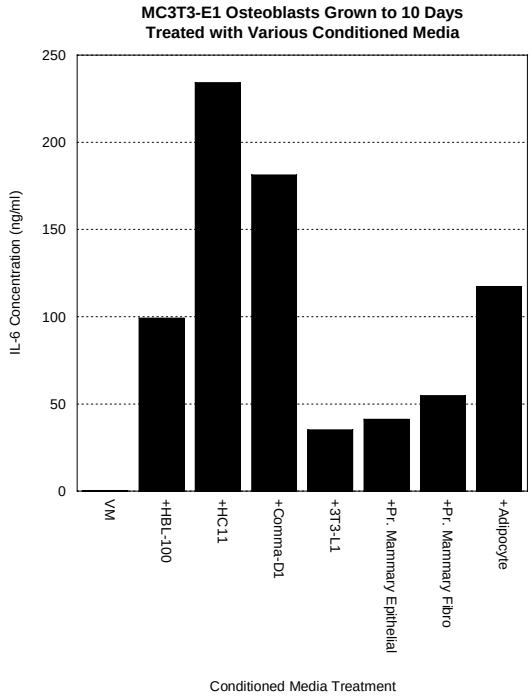




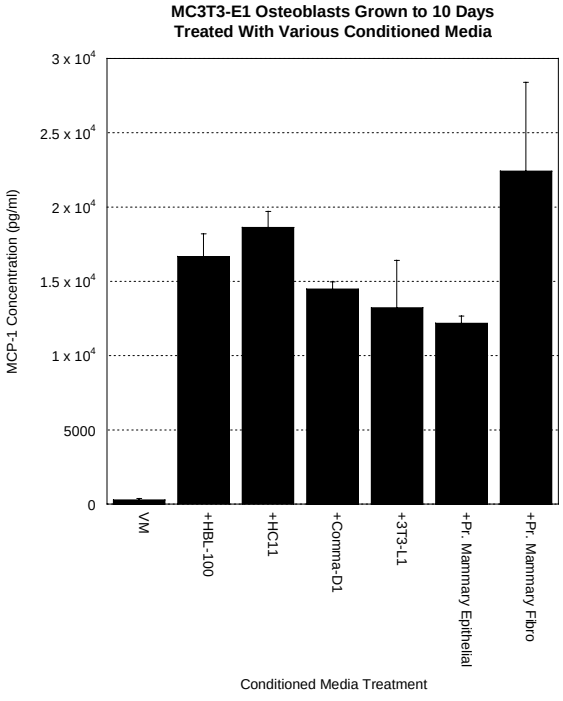
Murine MC3T3-E1 osteoblasts (grown to A) 10 or B) 26 days) were incubated with 0 or 50% conditioned medium from MDA-231 breast cancer cells for 24 hrs. Murine IL-6 and MCP-1 in the medium were quantified using standard ELISAs.

Figure 6.: Cytokine expression of MC3T3-E1 osteoblasts treated with 0 (VM) or 50% conditioned medium from various cell types.

A.



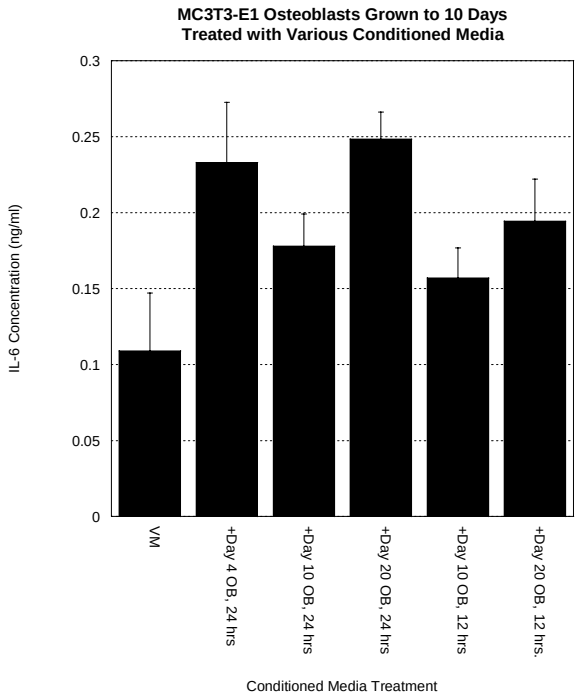
B.



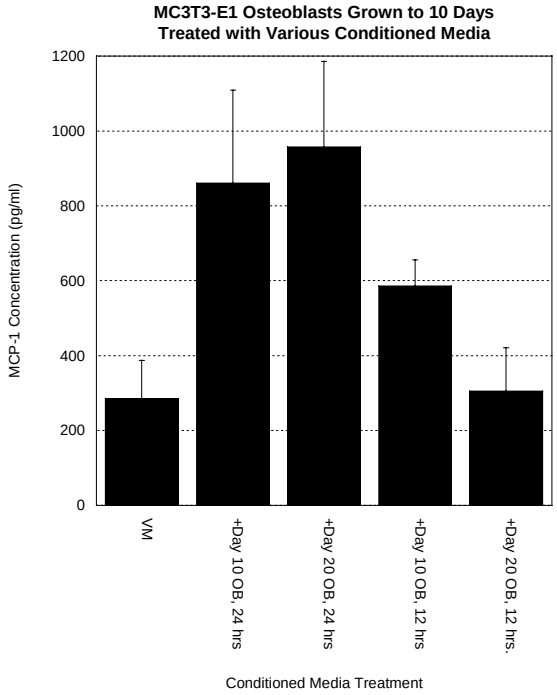
Murine MC3T3-E1 osteoblasts grown to 10 days old were incubated with 0 or 50% conditioned medium from non-metastatic cells for 24 hrs. Murine IL-6 (A) and MCP-1 (B) in the medium were quantified using standard ELISAs.

Figure 7.: Cytokine expression of MC3T3-E1 osteoblasts treated with 0 (VM) or 50% osteoblast conditioned medium.

A.



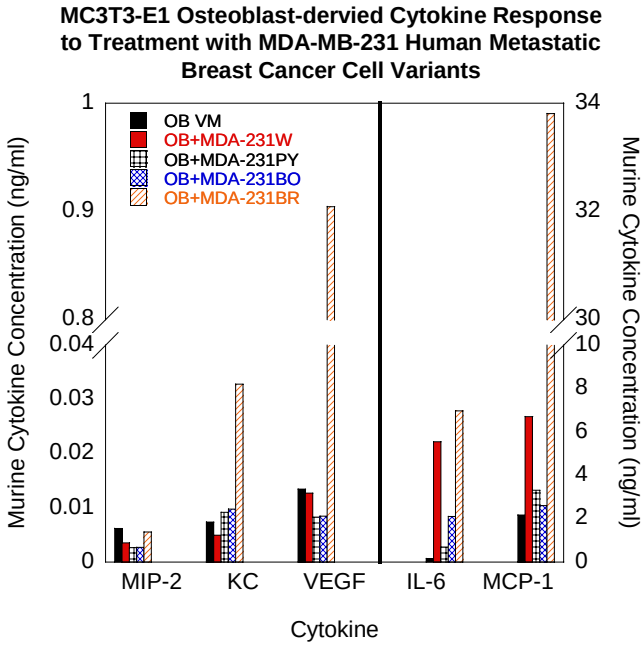
B.



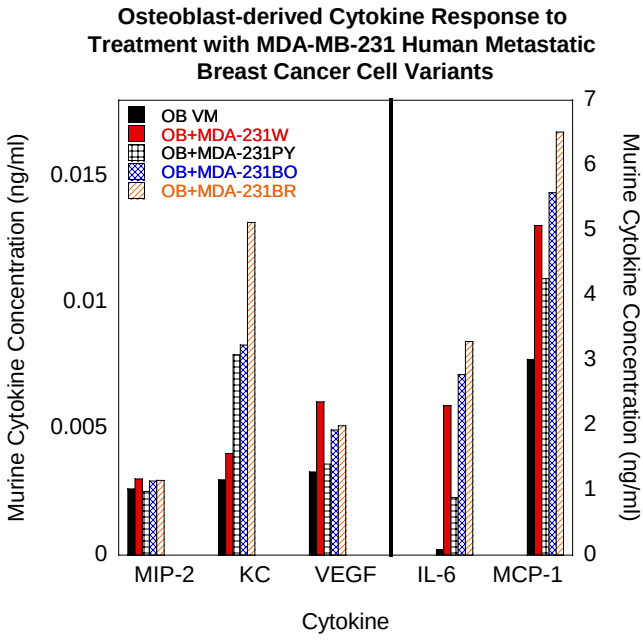
Murine MC3T3-E1 osteoblasts grown to 10 days were incubated with 0 or 50% conditioned medium from MC3T3-E1 osteoblast cells for 24 hrs. Murine IL-6 (A) and MCP-1 (B) in the medium were quantified using standard ELISAs.

Figure 8.: Murine MC3T3-E1 cytokine response to treatment with 0 (VM) or 50% conditioned medium from MDA-231 human metastatic breast cancer cell variants.

A.

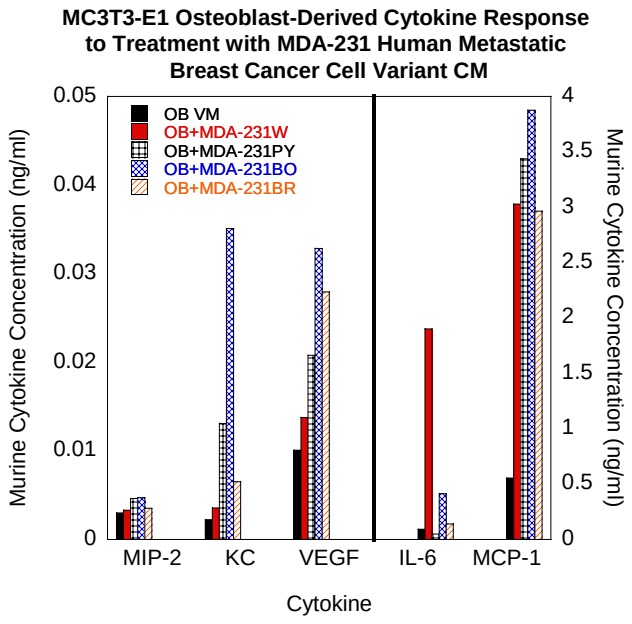


B.

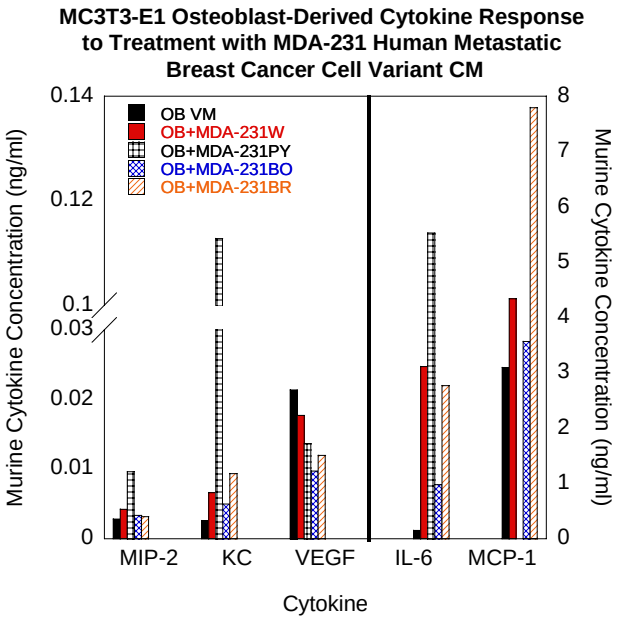


Day 10

C.



D.

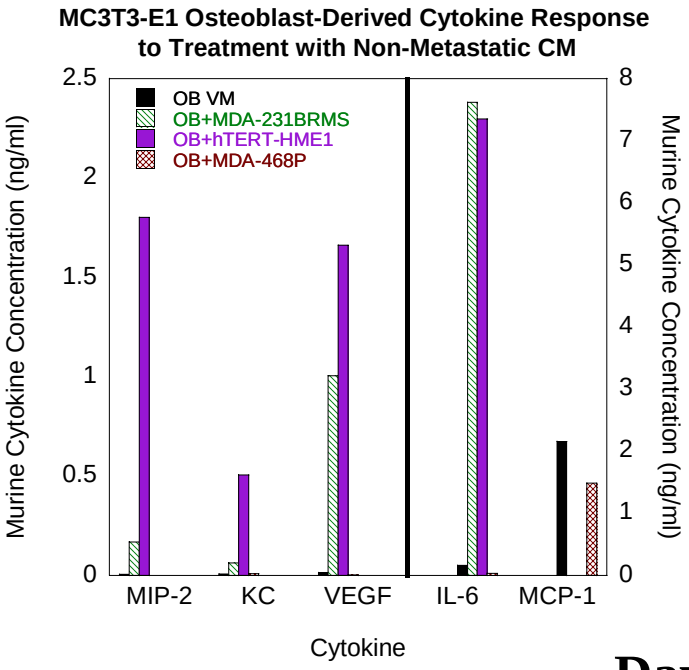


Day 20

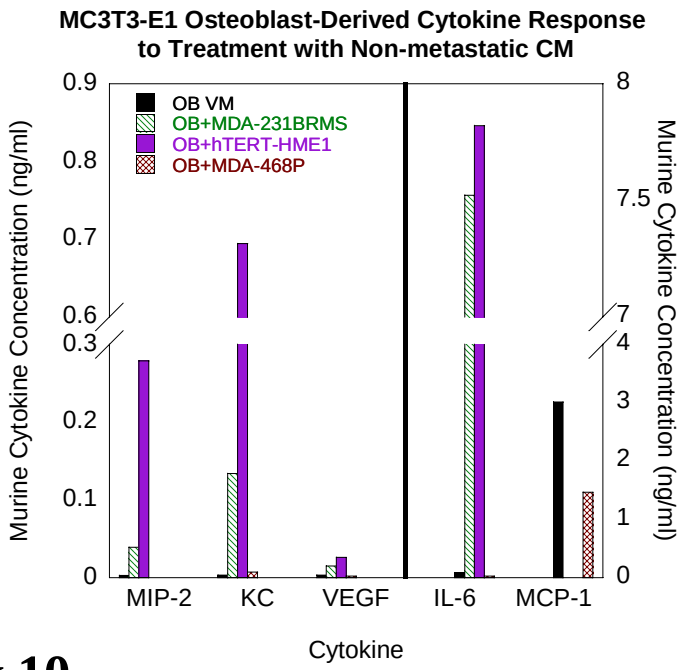
Murine MC3T3-E1 osteoblasts grown to 10 (A and B) or 20 days (C and D) were incubated with 0 or 50% conditioned medium from MDA-231 breast cancer cells for 24 hrs. Cytokines in the medium were quantified using Bio-Rad Bio-Plex™ Murine Cytokine quantification array. Graphs A-D represent treatment with individual batches of CM.

Figure 9.: Murine MC3T3-E1 cytokine response to treatment with 0 (VM) or 50% conditioned medium from human non-metastatic cell variants.

A.

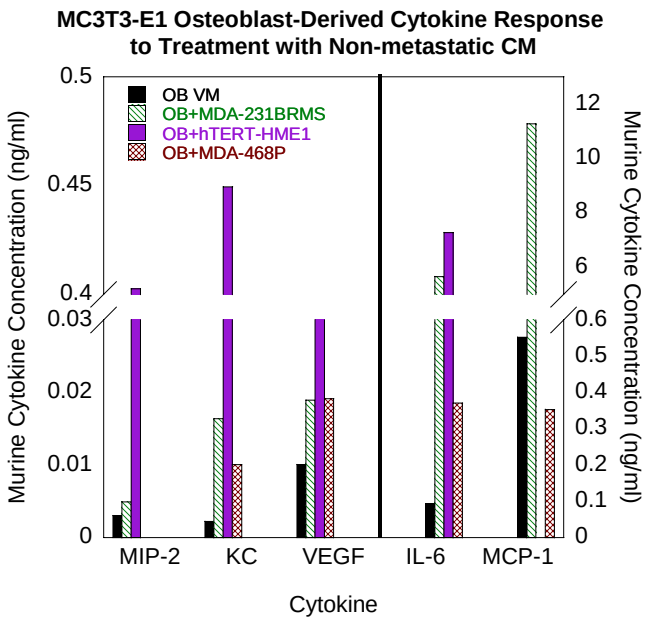


B.

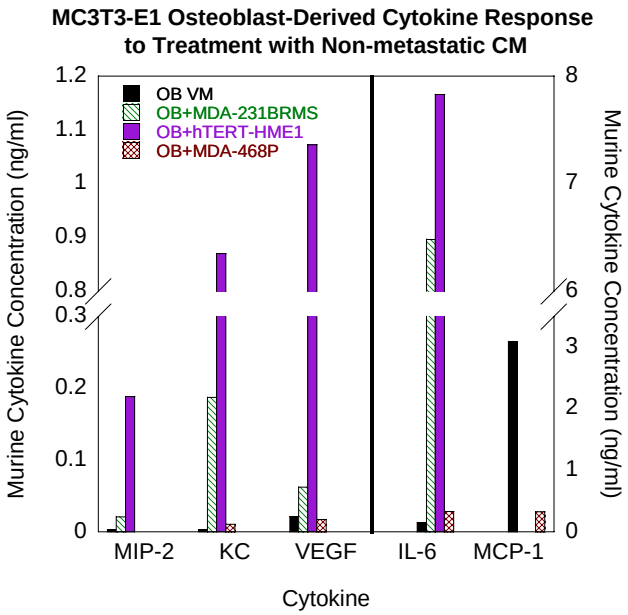


Day 10

C.



D.



Day 20

Murine MC3T3-E1 osteoblasts grown to 10 (A and B) or 20 days (C and D) were incubated with 0 or 50% conditioned medium from human non-metastatic cell variants for 24 hrs. Cytokines in the medium were quantified using Bio-Rad Bio-Plex™ Murine Cytokine quantification array. Graphs A-D represent treatment with individual batches of CM.

Figure 10: Illustration of Transwell Plate Assay Experimental Set-up

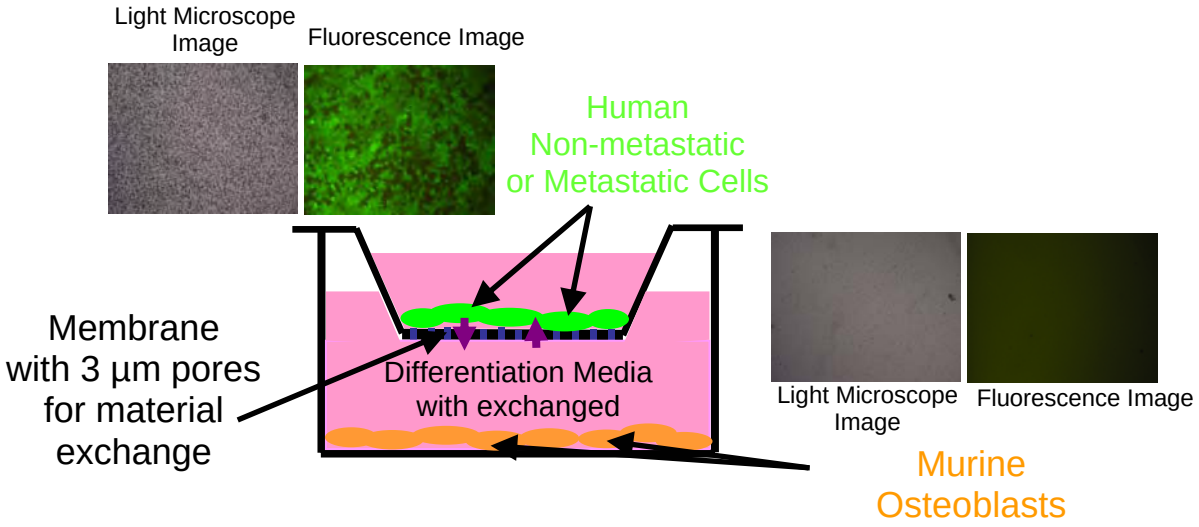
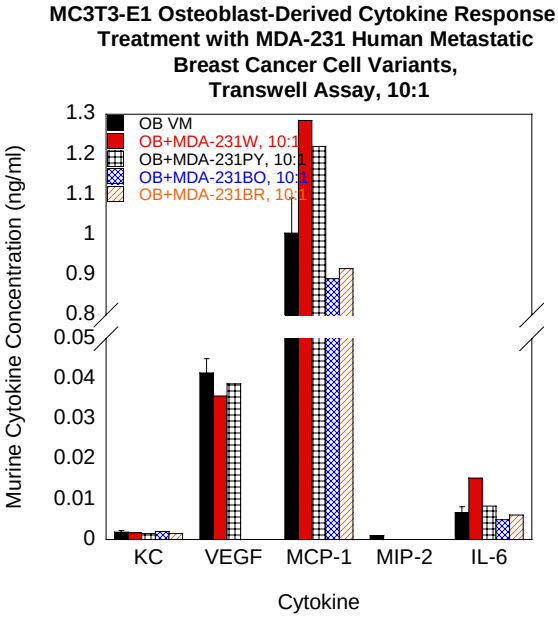
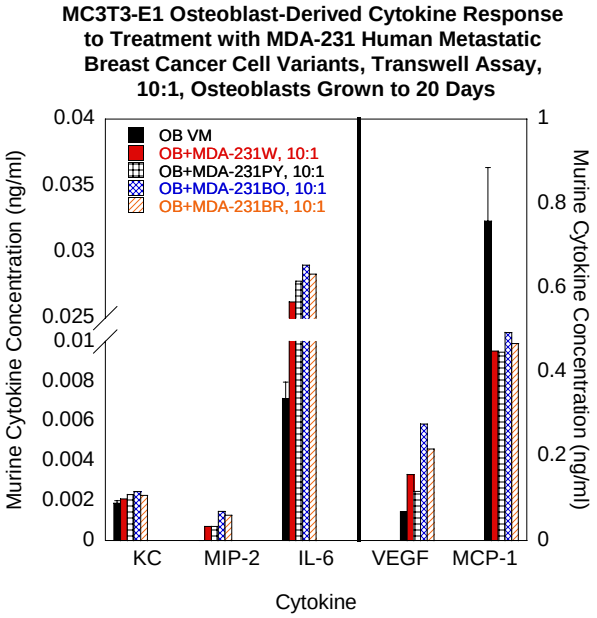


Figure 11.: Murine MC3T3-E1 cytokine response to treatment with 10:1 MDA-231 Human Metastatic Breast Cancer Cell Variants in a Transwell System.

A.



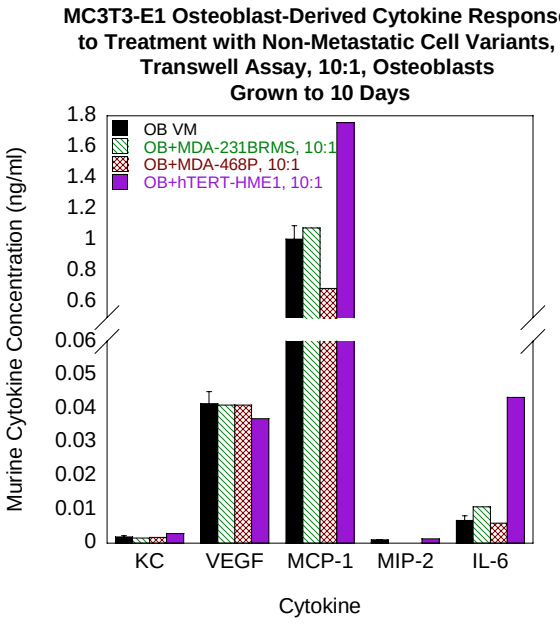
B.



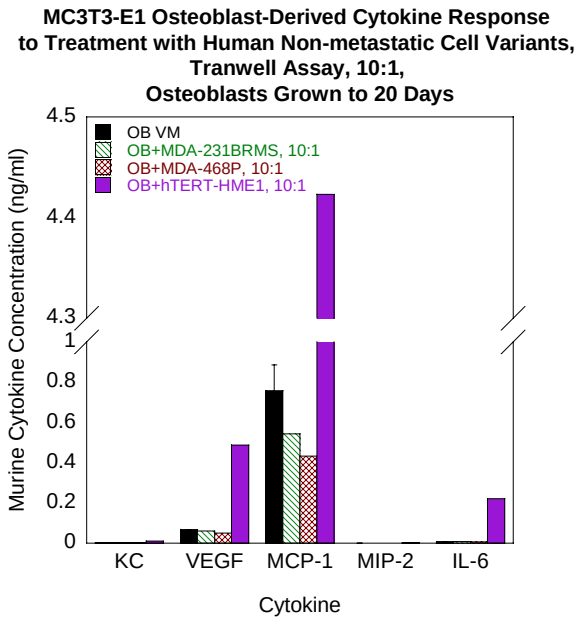
MC3T3-E1 murine osteoblasts grown to A) 10 or B) 20 days were incubated in a transwell system with MDA-231 human metastatic breast cancer cell variants for an additional 3 days. Osteoblast-derived murine cytokine concentrations were quantified using a murine Bio-Rad Bio-Plex.

Figure 12.: Murine MC3T3-E1 cytokine response to treatment with 10:1 MDA-231 Human Non-Metastatic Cell Variants in a Transwell System.

A.



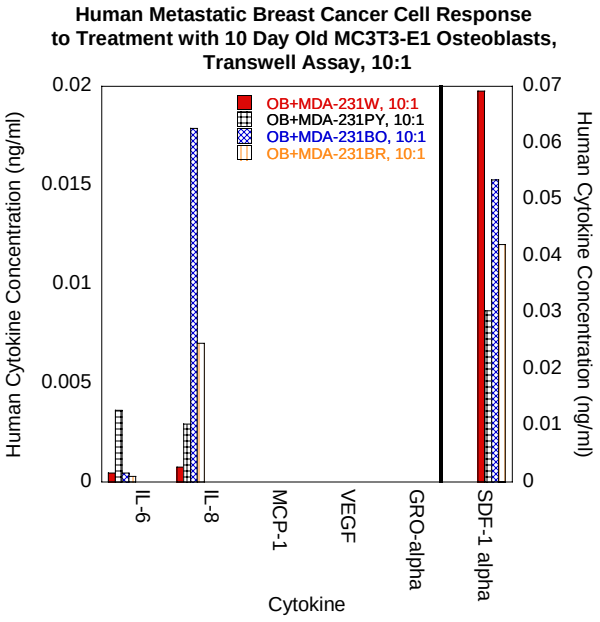
B.



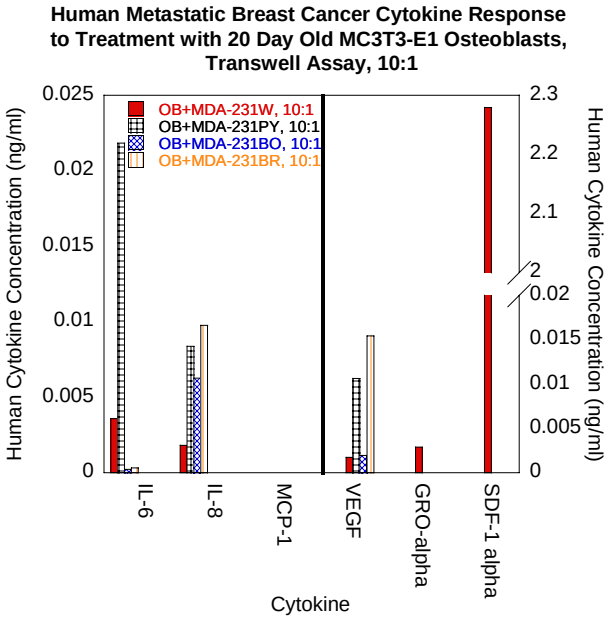
MC3T3-E1 murine osteoblasts grown to A) 10 or B) 20 days were incubated in a transwell system with MDA-231 human non-metastatic cell variants for an additional 3 days. Osteoblast-derived murine cytokine concentrations were quantified using a murine Bio-Rad Bio-Plex.

Figure 13.: Human Metastatic Breast Cancer Cell Variant Response to Murine MC3T3-E1 cytokine treatment in a Transwell System.

A.



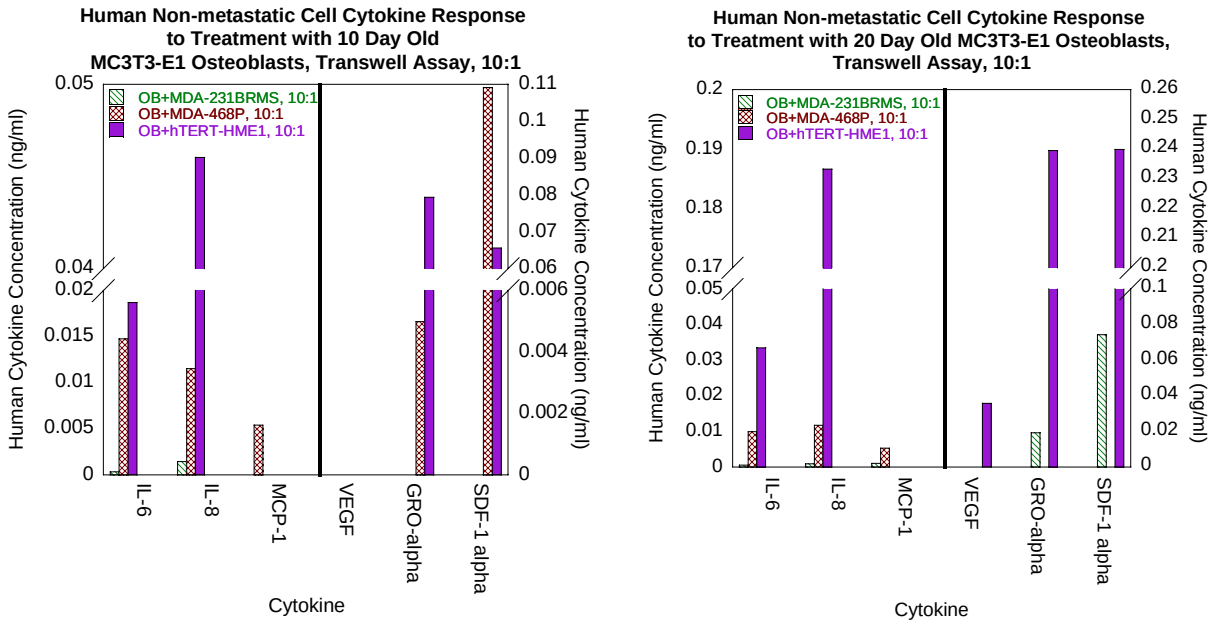
B.



MC3T3-E1 murine osteoblasts grown to A) 10 or B) 20 days were incubated in a transwell system with MDA-231 human metastatic breast cancer cell variants for an additional 3 days. Breast Cancer Cell-derived Human cytokine concentrations were quantified using a human Bio-Rad Bio-Plex.

Figure 14.: Human Non-Metastatic Cell Variant Response to Murine MC3T3-E1 cytokine treatment in a Transwell System.

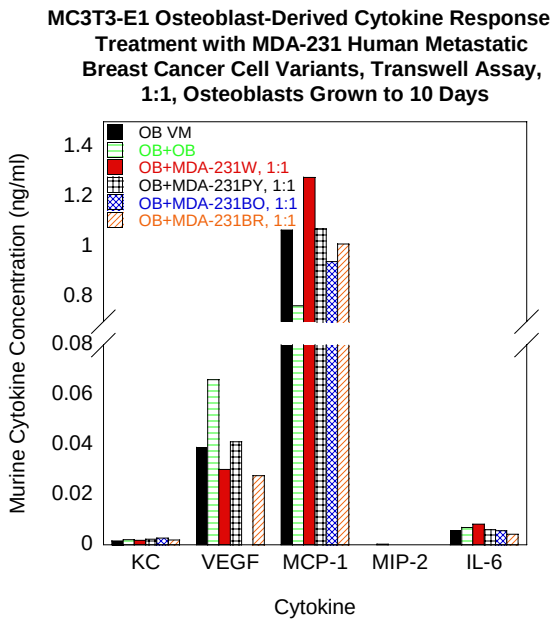
A. B.



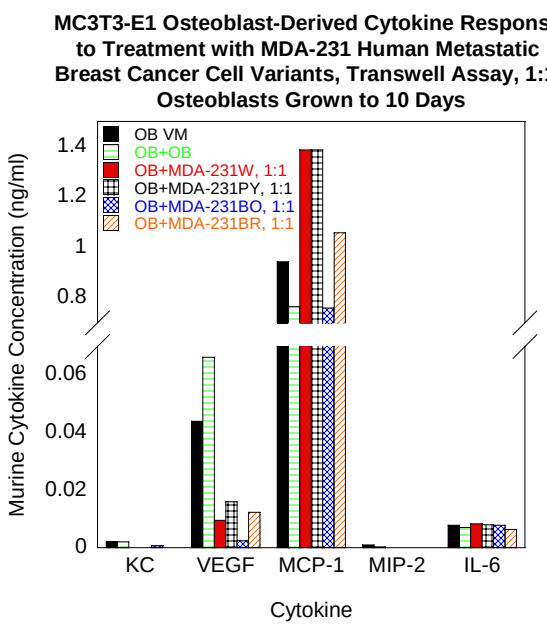
MC3T3-E1 murine osteoblasts grown to A) 10 or B) 20 days were incubated in a transwell system with human non-metastatic breast cancer cell variants at a ratio of 10:1 (osteoblast : non-osteoblasts) for an additional 3 days. Breast Cancer Cell-derived Human cytokine concentrations were quantified using a human Bio-Rad Bio-Plex.

Figure 15.: Murine MC3T3-E1 cytokine response to treatment with 1:1 MDA-231 Human Metastatic Breast Cancer Cell Variants in a Transwell System.

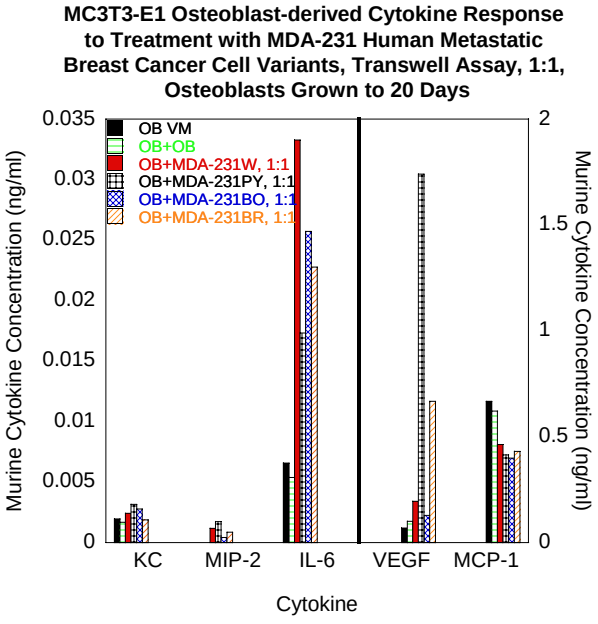
A.



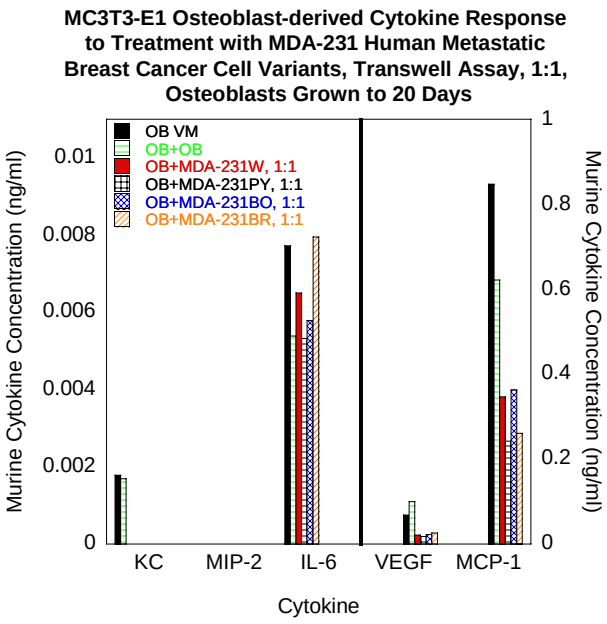
B.



C.



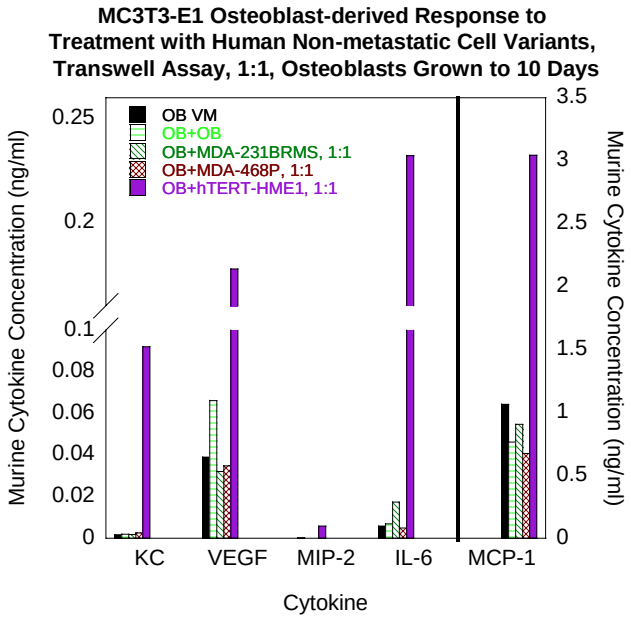
D.



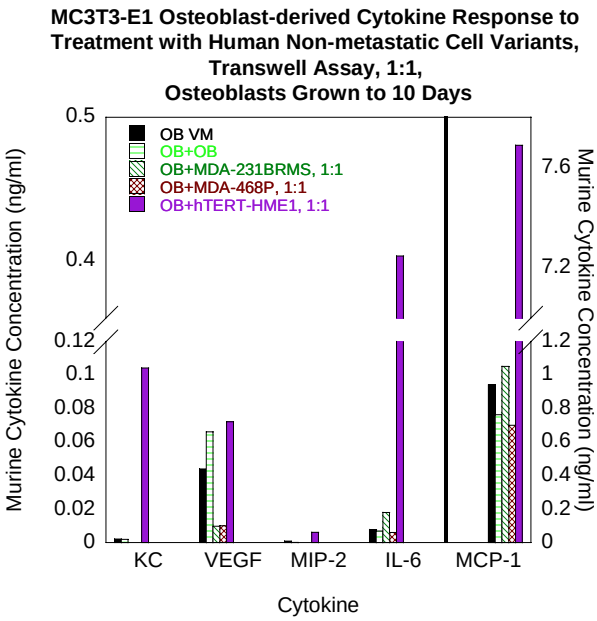
MC3T3-E1 murine osteoblasts grown to A-B) 10 or C-D) 20 days were incubated in a transwell system with MDA-231 human metastatic breast cancer cell variants for an additional 3 days. Osteoblast-derived murine cytokine concentrations were quantified using a murine Bio-Rad Bio-Plex.

Figure 16.: Murine MC3T3-E1 cytokine response to treatment with 1:1 Human Non-Metastatic Cell Variants in a Transwell System.

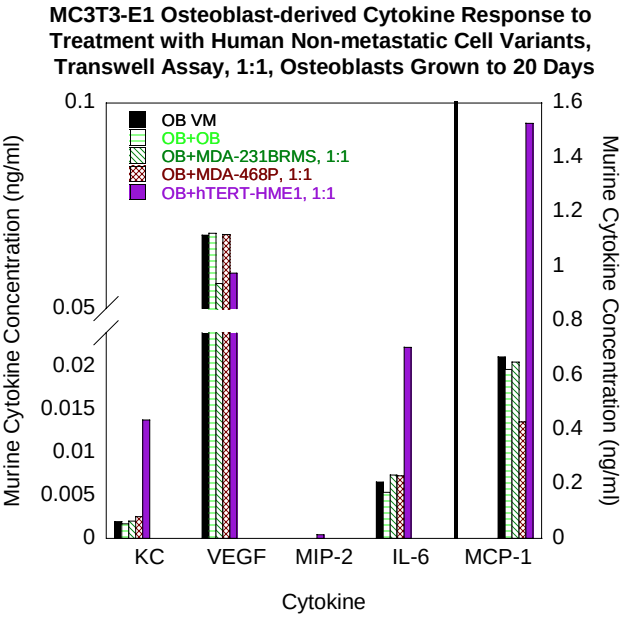
A.



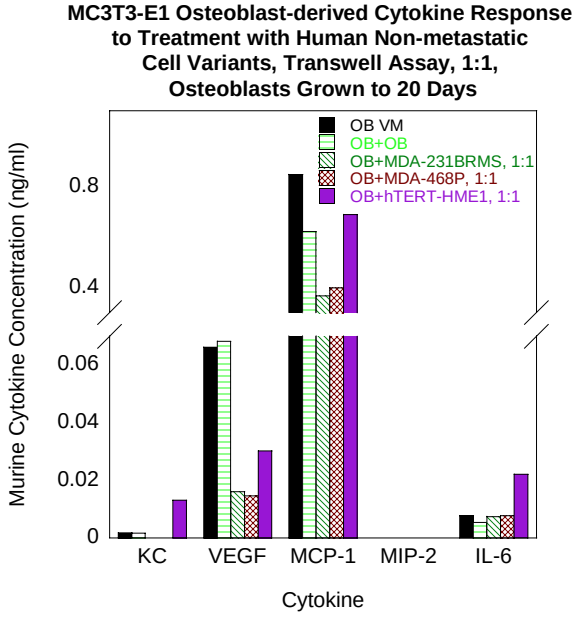
B.



C.



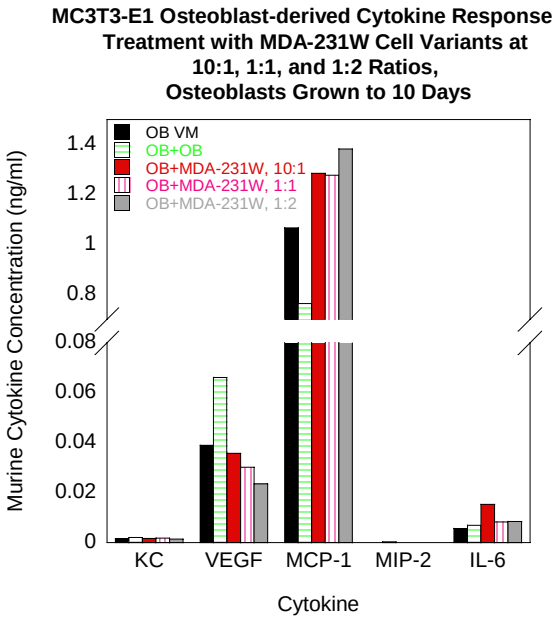
D.



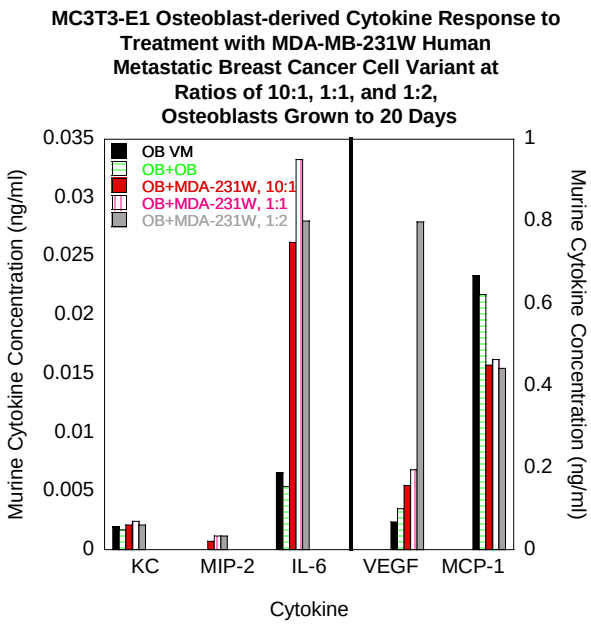
MC3T3-E1 murine osteoblasts grown to A-B) 10 or C-D) 20 days were incubated in a transwell system with human non-metastatic cell variants for an additional 3 days. Osteoblast-derived murine cytokine concentrations were quantified using a murine Bio-Rad Bio-Plex.

Figure 17.: Murine MC3T3-E1 cytokine response to treatment with 1:1 MDA-MB-231W Human Metastatic Breast Cancer Cells at Ratios of 10:1, 1:1, and 1:2 in a Transwell System.

A.



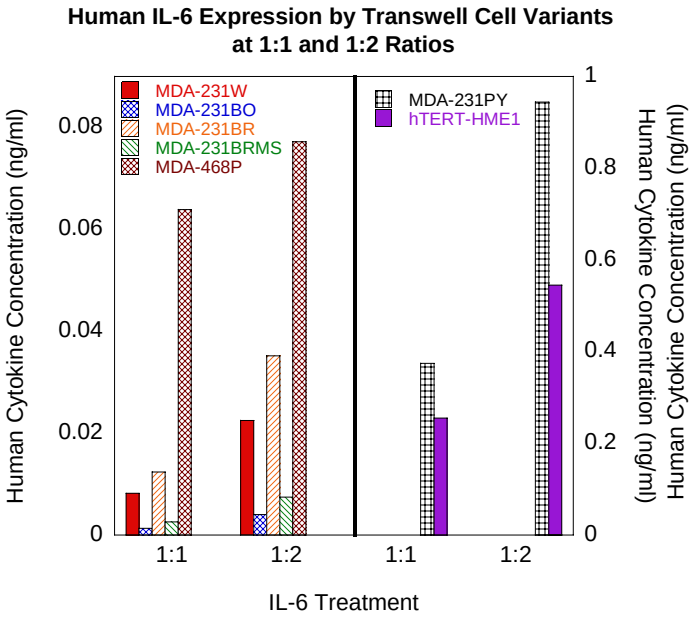
B.



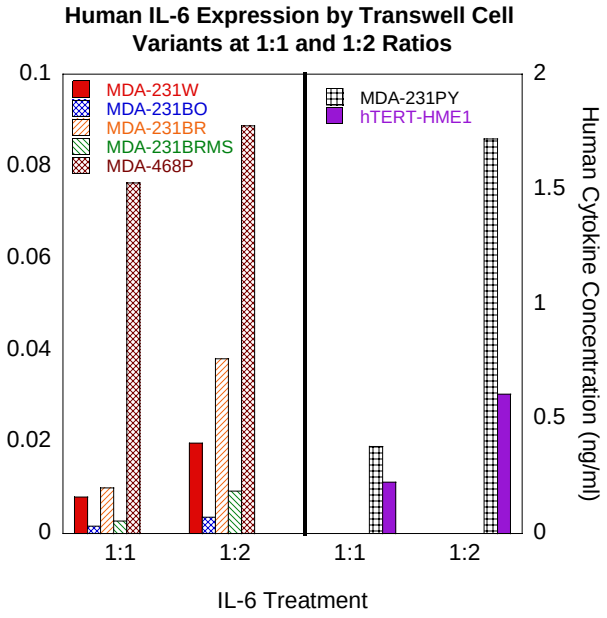
MC3T3-E1 murine osteoblasts grown to A) 10 or B) 20 days were incubated in a transwell system with MDA-MB-231W human metastatic breast cancer cells for an additional 3 days. Osteoblast-derived murine cytokine concentrations were quantified using a murine Bio-Rad Bio-Plex.

Figure 18.: Human Transwell Cell Variant Cytokine Response to Treatment with Murine MC3T3-E1 Osteoblasts 1:1 and 1:2 in a Transwell System.

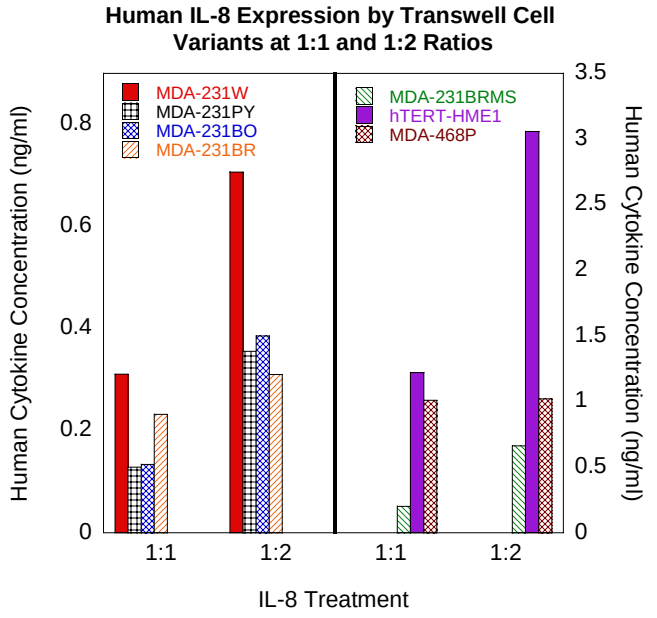
A.



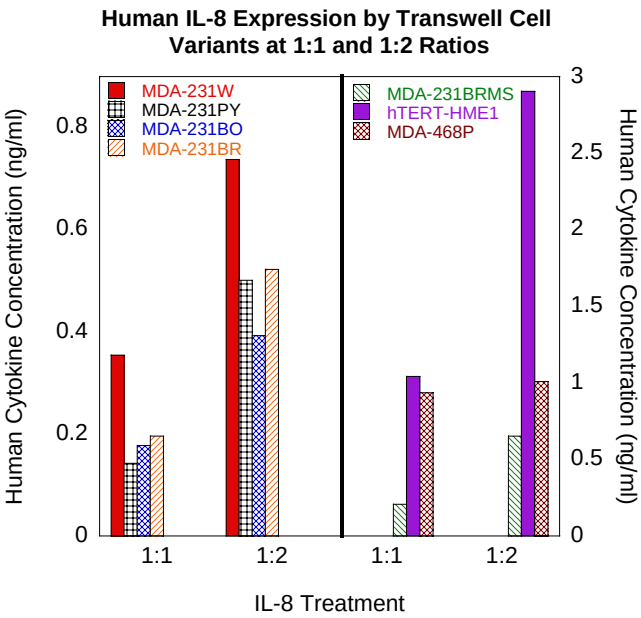
B.



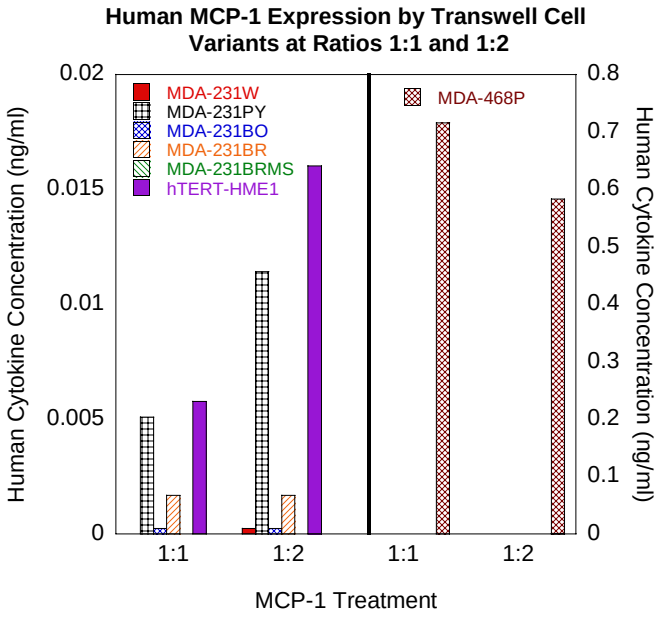
C.



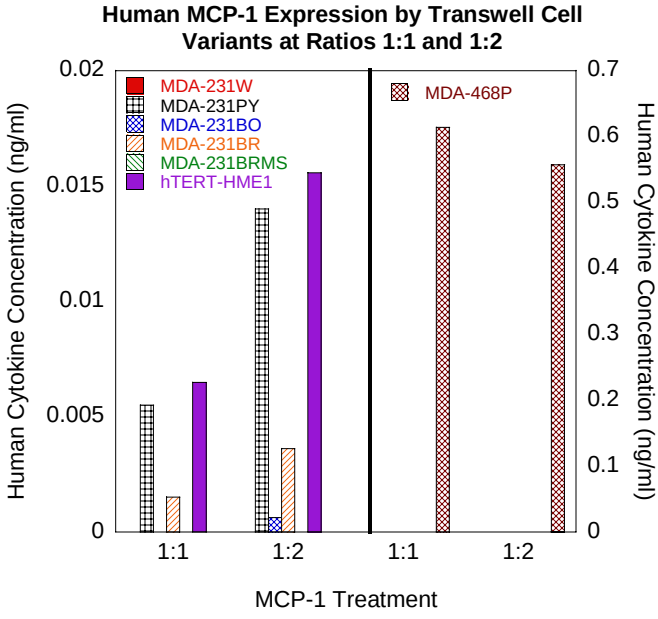
D.



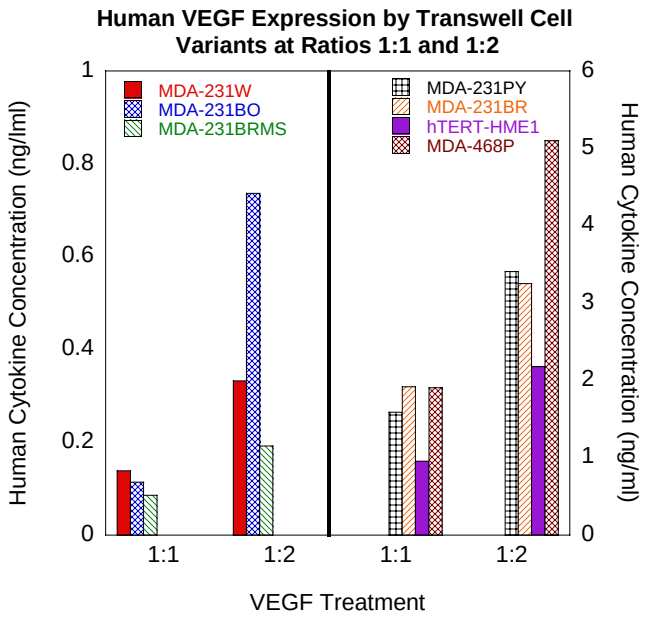
E.


Human MCP-1 Expression by Transwell Cell Variants at Ratios 1:1 and 1:2
Human Cytokine Concentration (ng/ml)
Human Cytokine Concentration (ng/ml)
MDA-231W
MDA-231PY
MDA-231BO
MDA-231BR
MDA-231BRMS
hTERT-HME1
MDA-468P
0.02
0.015
0.01
0.005
0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1:1
1:2
1:1
1:2
MCP-1 Treatment

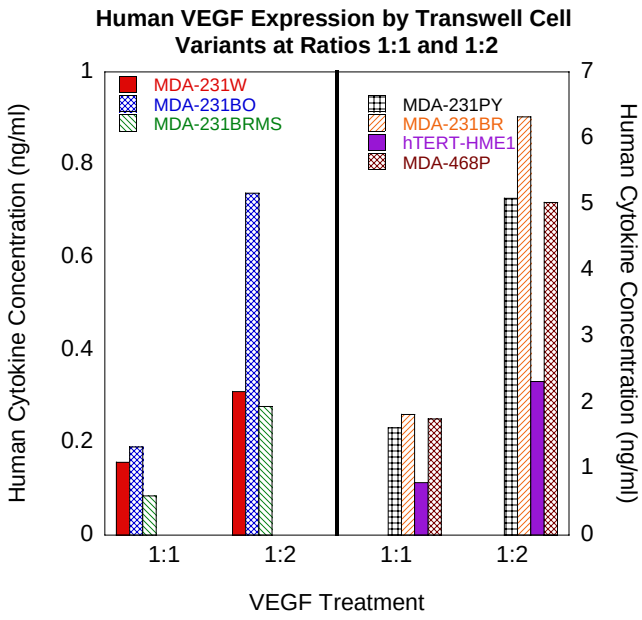
F.


Human MCP-1 Expression by Transwell Cell Variants at Ratios 1:1 and 1:2
Human Cytokine Concentration (ng/ml)
Human Cytokine Concentration (ng/ml)
MDA-231W
MDA-231PY
MDA-231BO
MDA-231BR
MDA-231BRMS
hTERT-HME1
MDA-468P
0.02
0.015
0.01
0.005
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1:1
1:2
1:1
1:2
MCP-1 Treatment

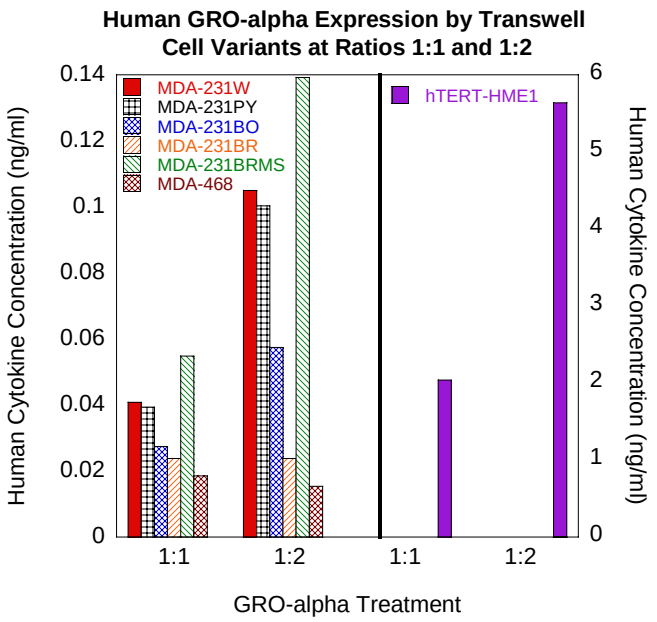
G.


Human VEGF Expression by Transwell Cell Variants at Ratios 1:1 and 1:2
Human Cytokine Concentration (ng/lml)
Human Cytokine Concentration (ng/ml)
MDA-231W
MDA-231BO
MDA-231BRMS
MDA-231PY
MDA-231BR
hTERT-HME1
MDA-468P
1
0.8
0.6
0.4
0.2
0
6
5
4
3
2
1
0
1:1
1:2
1:1
1:2
VEGF Treatment

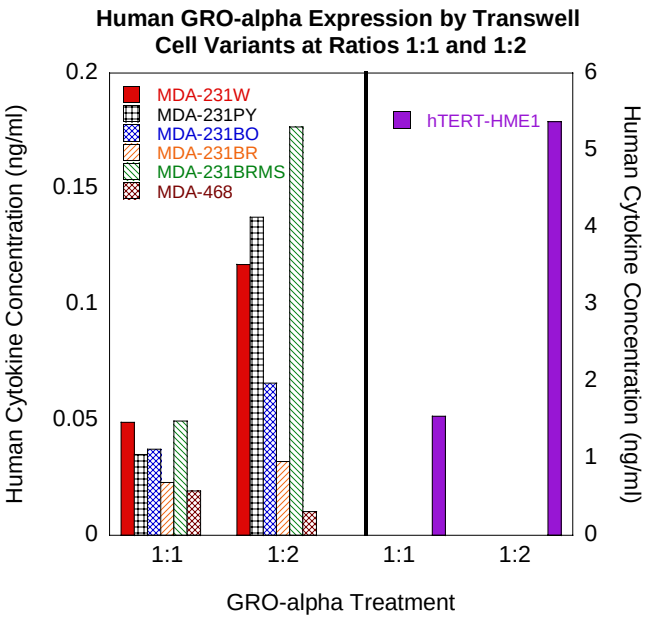
H.


Human VEGF Expression by Transwell Cell Variants at Ratios 1:1 and 1:2
Human Cytokine Concentration (ng/ml)
Human Cytokine Concentration (ng/ml)
MDA-231W
MDA-231BO
MDA-231BRMS
MDA-231PY
MDA-231BR
hTERT-HME1
MDA-468P
1
0.8
0.6
0.4
0.2
0
7
6
5
4
3
2
1
0
1:1
1:2
1:1
1:2
VEGF Treatment

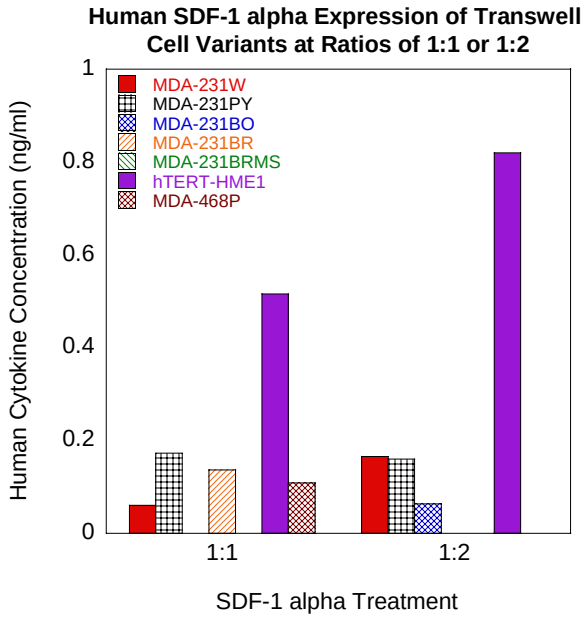
I.



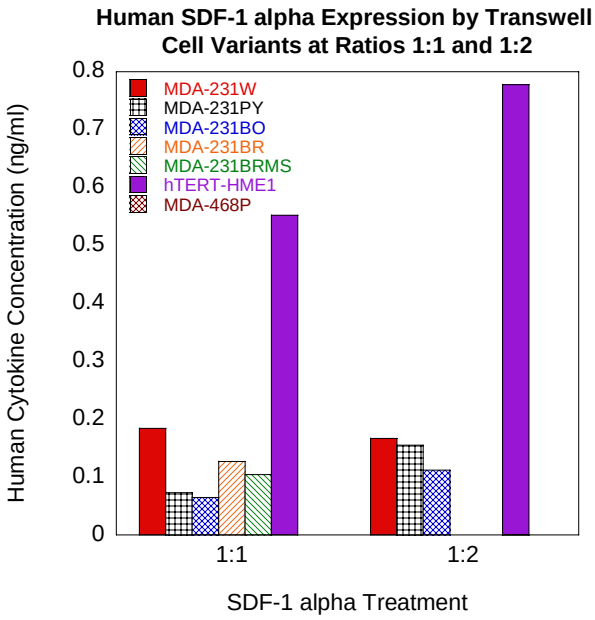
J.



K.



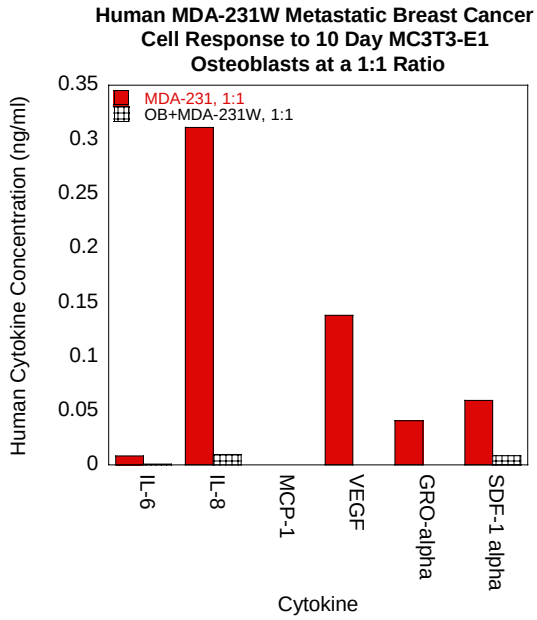
L.



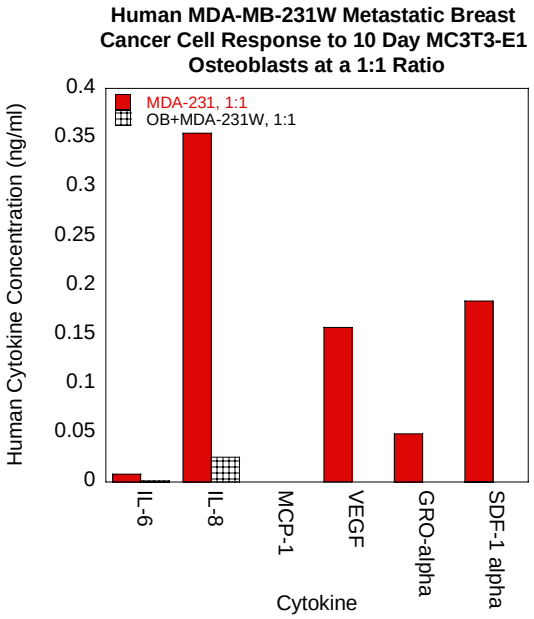
Human transwell cell variants were plated in the insert of a transwell system at ratios of 1:1 or 1:2 by themselves and incubated for 3 days. Breast Cancer Cell-derived Human cytokine concentrations were quantified using a human Bio-Rad Bio-Plex (A. Set A IL-6; B. Set B, IL-6; C. Set A IL-8; D. Set B IL-8; E. Set A MCP-1; F. Set B MCP-1; G. Set A VEGF; H. Set B VEGF; I. Set A GRO-alpha; J. Set B GRO-alpha; K. Set A SDF-1 Alpha; L. Set B SDF-1 alpha).

Figure 19.: Human Transwell Cell Variant Cytokine Response to Treatment with Murine MC3T3-E1 Osteoblasts at a 1:1 Ratio in a Transwell System.

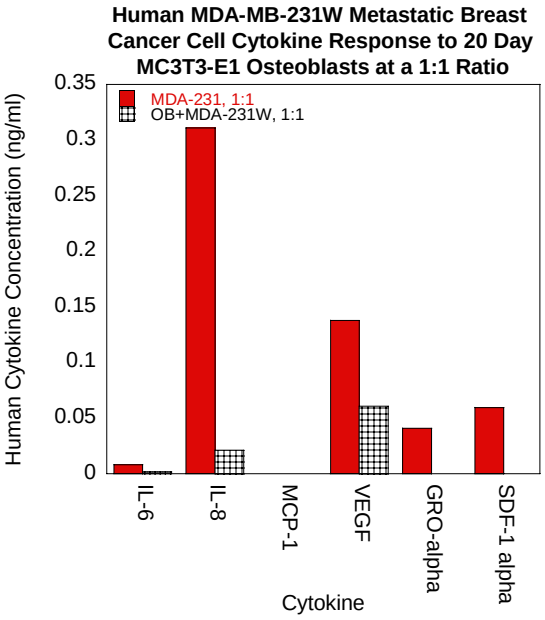
A.



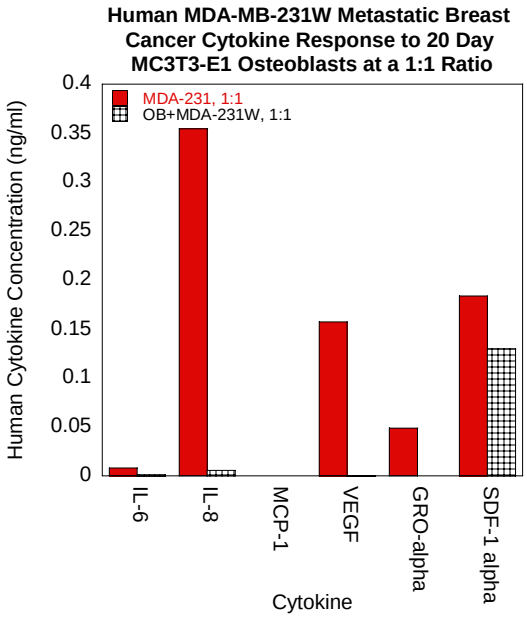
B.



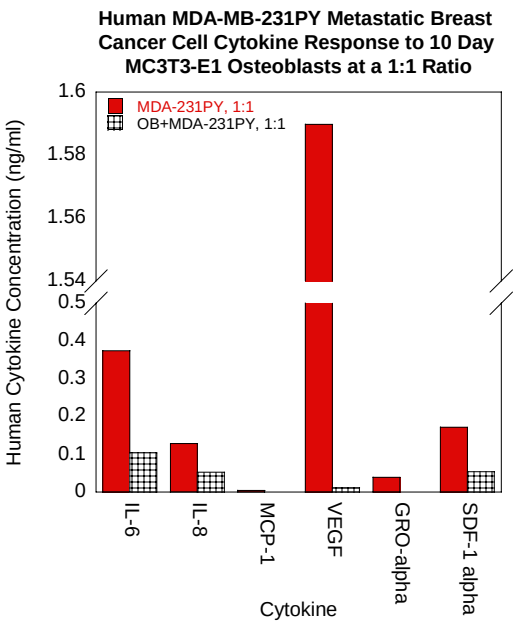
C.



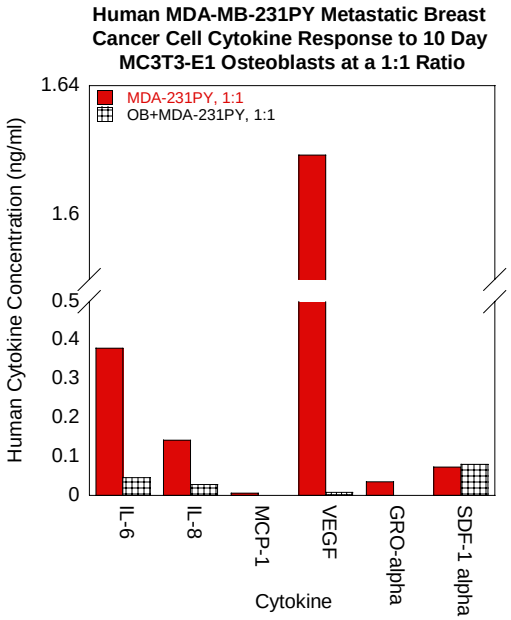
D.



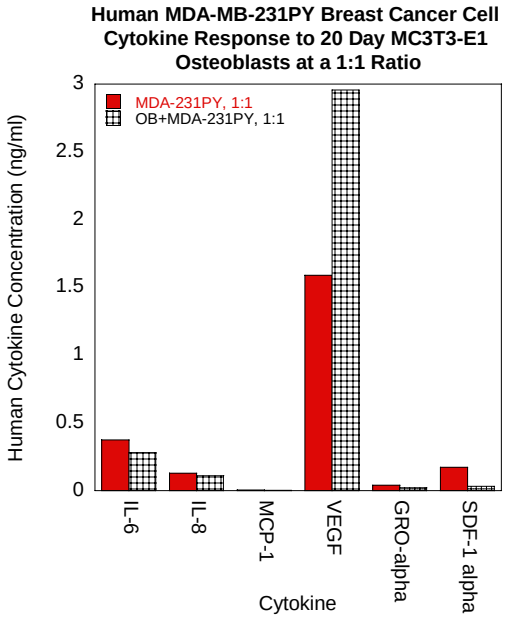
E.


Human MDA-MB-231PY Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231PY, 1:1
OB+MDA-231PY, 1:1
Human Cytokine Concentration (ng/ml)
1.6
1.58
1.56
1.54
0.5
0.4
0.3
0.2
0.1
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

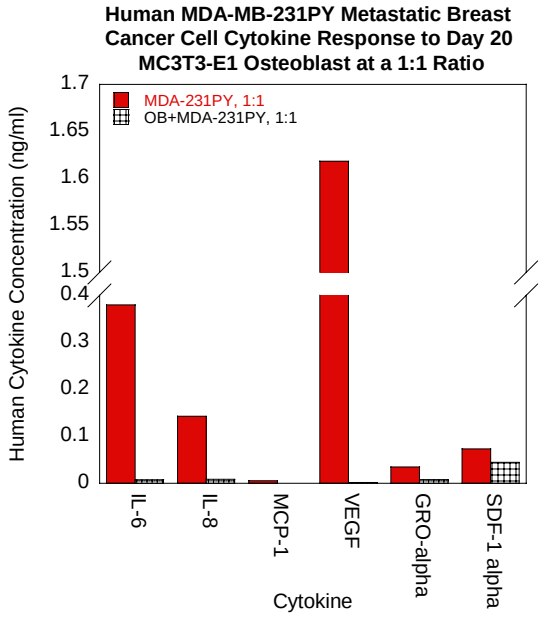
F.


Human MDA-MB-231PY Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231PY, 1:1
OB+MDA-231PY, 1:1
Human Cytokine Concentration (ng/ml)
1.64
1.6
0.5
0.4
0.3
0.2
0.1
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

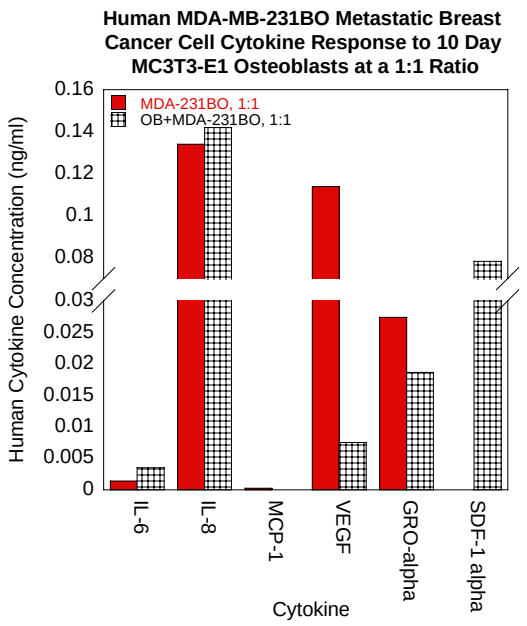
G.


Human MDA-MB-231PY Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231PY, 1:1
OB+MDA-231PY, 1:1
Human Cytokine Concentration (ng/ml)
3
2.5
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

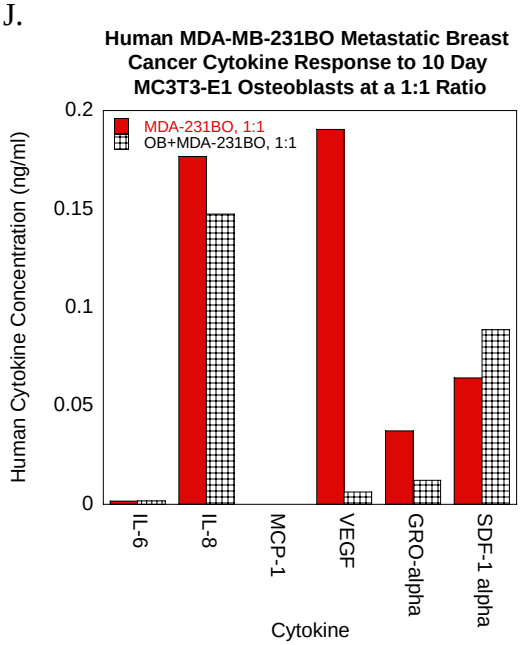
H.


Human MDA-MB-231PY Metastatic Breast Cancer Cell Cytokine Response to Day 20 MC3T3-E1 Osteoblast at a 1:1 Ratio
MDA-231PY, 1:1
OB+MDA-231PY, 1:1
Human Cytokine Concentration (ng/ml)
1.7
1.65
1.6
1.55
1.5
0.4
0.3
0.2
0.1
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

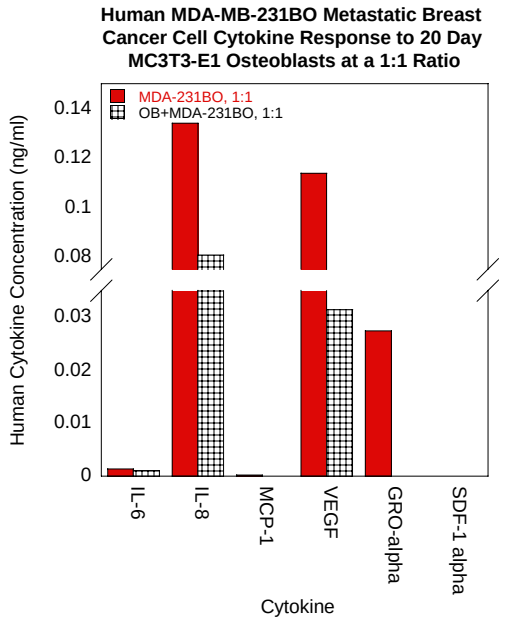
I.


Human MDA-MB-231BO Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BO, 1:1
OB+MDA-231BO, 1:1
Human Cytokine Concentration (ng/ml)
0.16
0.14
0.12
0.1
0.08
0.03
0.025
0.02
0.015
0.01
0.005
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

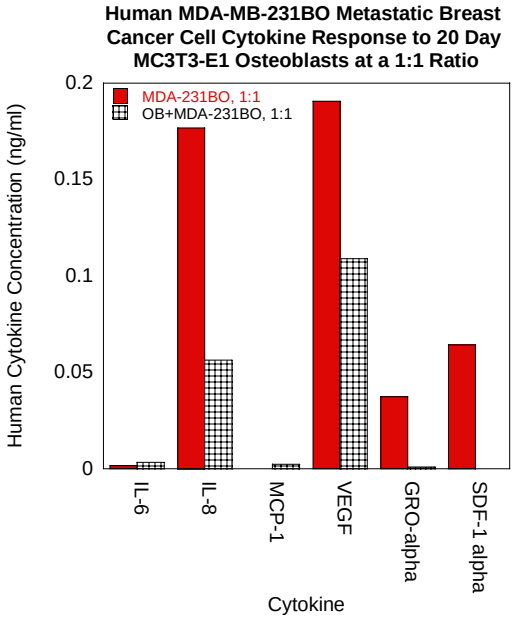
J.


Human MDA-MB-231BO Metastatic Breast Cancer Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BO, 1:1
OB+MDA-231BO, 1:1
Human Cytokine Concentration (ng/ml)
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

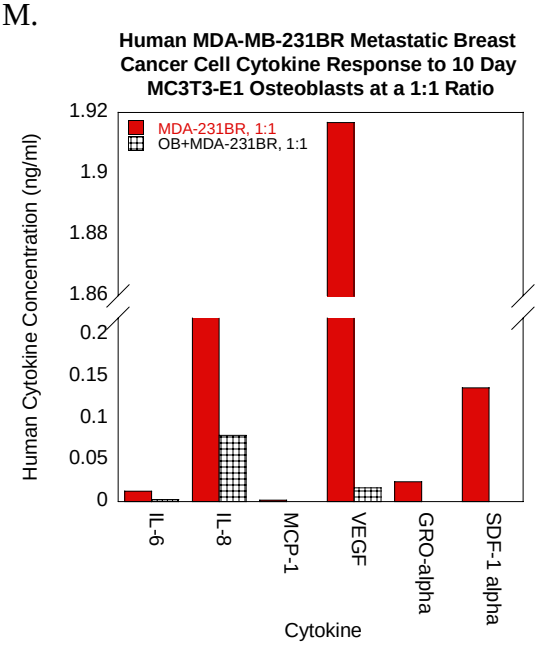
K.


Human MDA-MB-231BO Metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BO, 1:1
OB+MDA-231BO, 1:1
Human Cytokine Concentration (ng/ml)
0.14
0.12
0.1
0.08
0.03
0.02
0.01
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

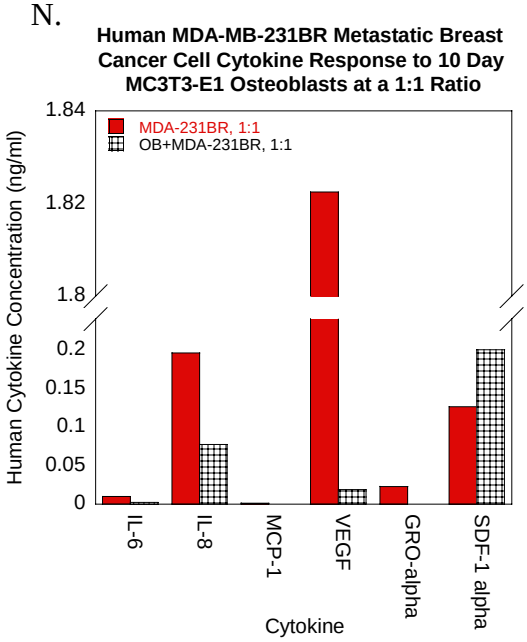
L.


Human MDA-MB-231BO Metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BO, 1:1
OB+MDA-231BO, 1:1
Human Cytokine Concentration (ng/ml)
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

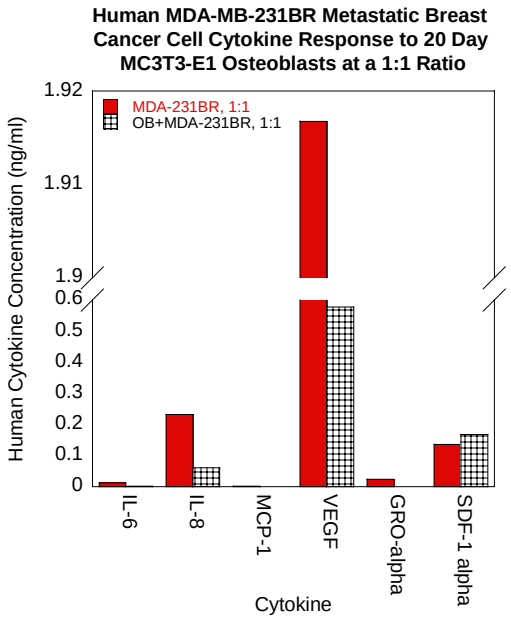
M.


Human MDA-MB-231BR Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BR, 1:1
OB+MDA-231BR, 1:1
Human Cytokine Concentration (ng/ml)
1.92
1.9
1.88
1.86
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

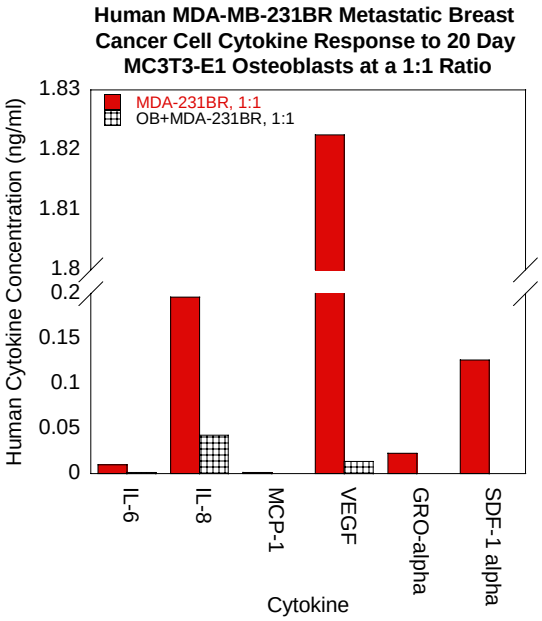
N.


Human MDA-MB-231BR Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BR, 1:1
OB+MDA-231BR, 1:1
Human Cytokine Concentration (ng/ml)
1.84
1.82
1.8
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

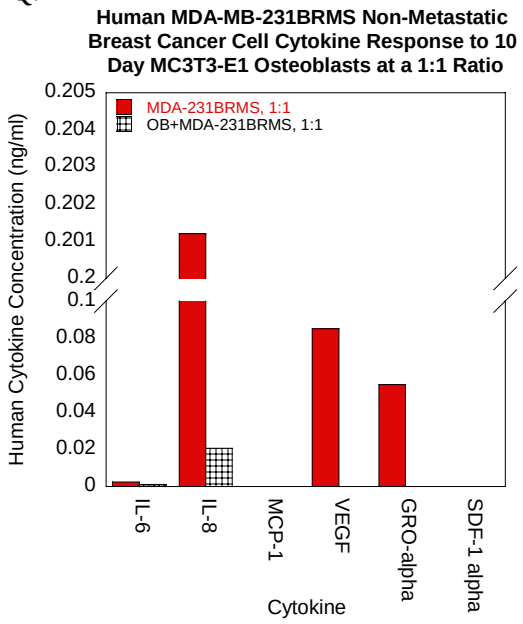
O.


Human MDA-MB-231BR Metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BR, 1:1
OB+MDA-231BR, 1:1
Human Cytokine Concentration (ng/ml)
1.92
1.91
1.9
0.6
0.5
0.4
0.3
0.2
0.1
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

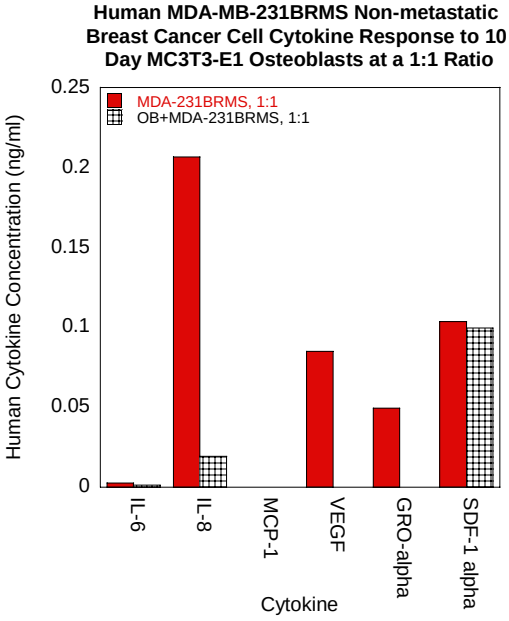
P.


Human MDA-MB-231BR Metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BR, 1:1
OB+MDA-231BR, 1:1
Human Cytokine Concentration (ng/ml)
1.83
1.82
1.81
1.8
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

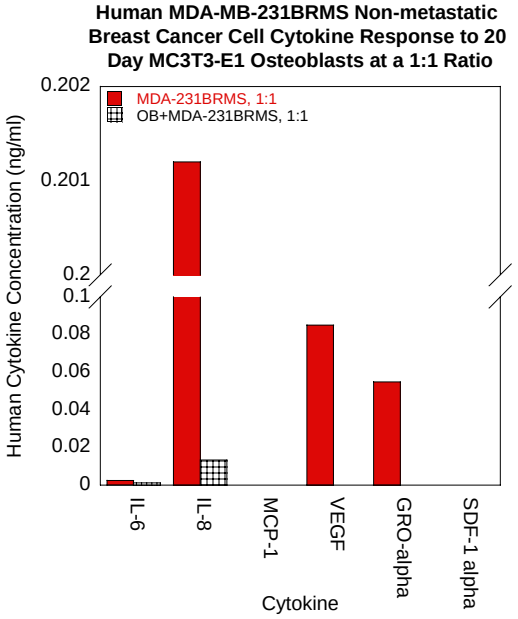
Q.


Human MDA-MB-231BRMS Non-Metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BRMS, 1:1
OB+MDA-231BRMS, 1:1
Human Cytokine Concentration (ng/ml)
0.205
0.204
0.203
0.202
0.201
0.2
0.1
0.08
0.06
0.04
0.02
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

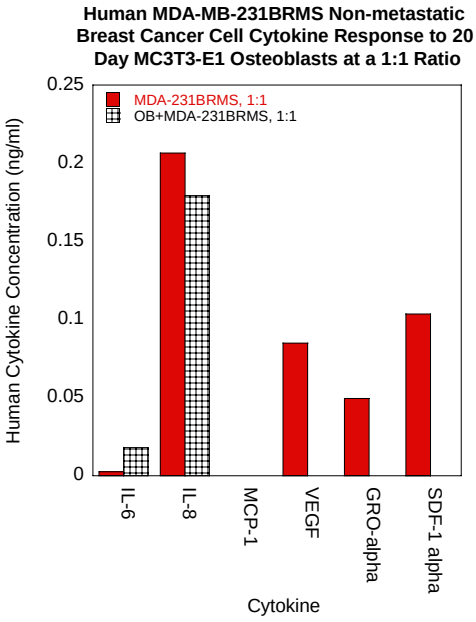
R.


Human MDA-MB-231BRMS Non-metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BRMS, 1:1
OB+MDA-231BRMS, 1:1
Human Cytokine Concentration (ng/ml)
0.25
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

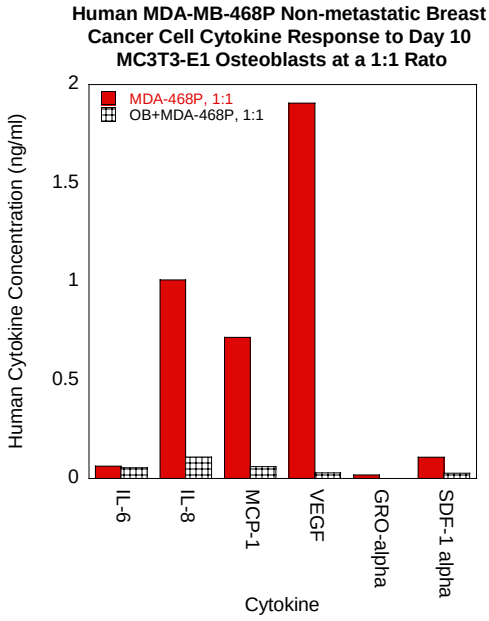
S.


Human MDA-MB-231BRMS Non-metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BRMS, 1:1
OB+MDA-231BRMS, 1:1
Human Cytokine Concentration (ng/ml)
0.202
0.201
0.2
0.1
0.08
0.06
0.04
0.02
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

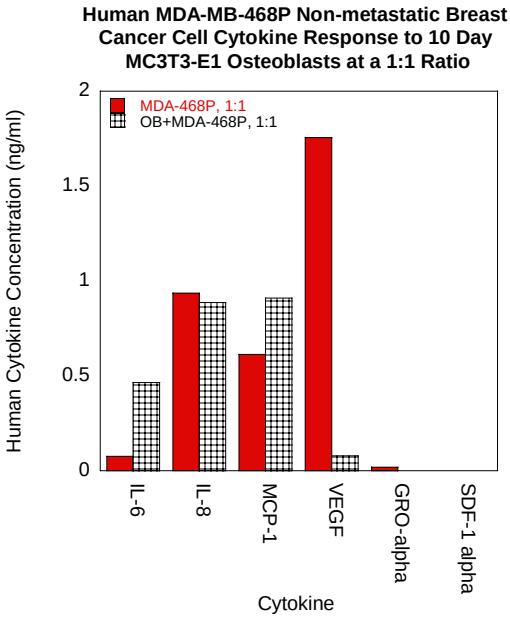
T.


Human MDA-MB-231BRMS Non-metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-231BRMS, 1:1
OB+MDA-231BRMS, 1:1
Human Cytokine Concentration (ng/ml)
0.25
0.2
0.15
0.1
0.05
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

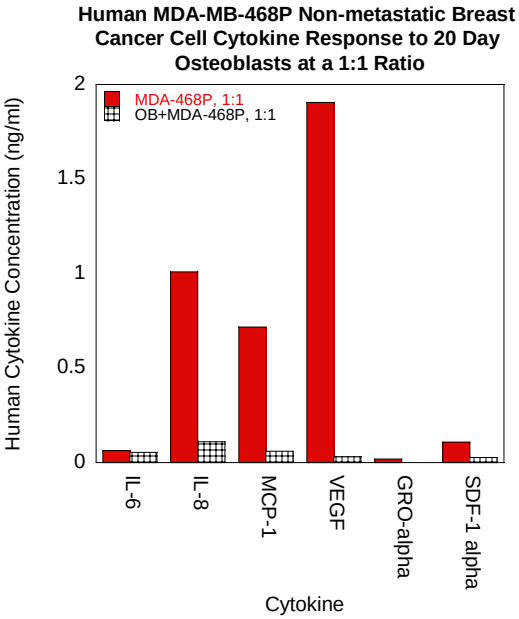
U.


Human MDA-MB-468P Non-metastatic Breast Cancer Cell Cytokine Response to Day 10 MC3T3-E1 Osteoblasts at a 1:1 Rato
MDA-468P, 1:1
OB+MDA-468P, 1:1
Human Cytokine Concentration (ng/ml)
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

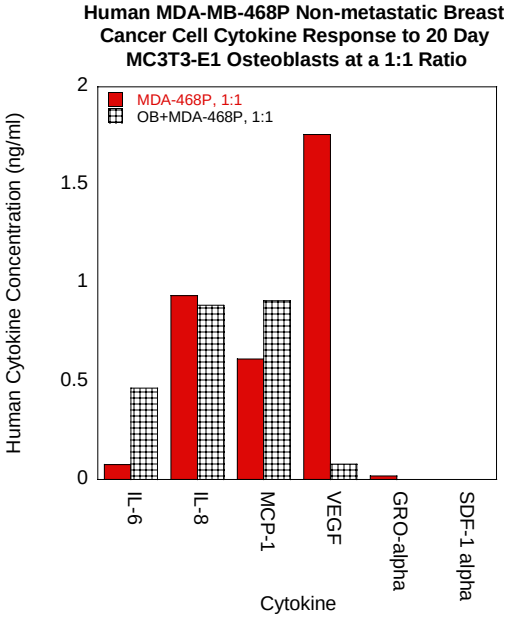
V.


Human MDA-MB-468P Non-metastatic Breast Cancer Cell Cytokine Response to 10 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-468P, 1:1
OB+MDA-468P, 1:1
Human Cytokine Concentration (ng/ml)
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

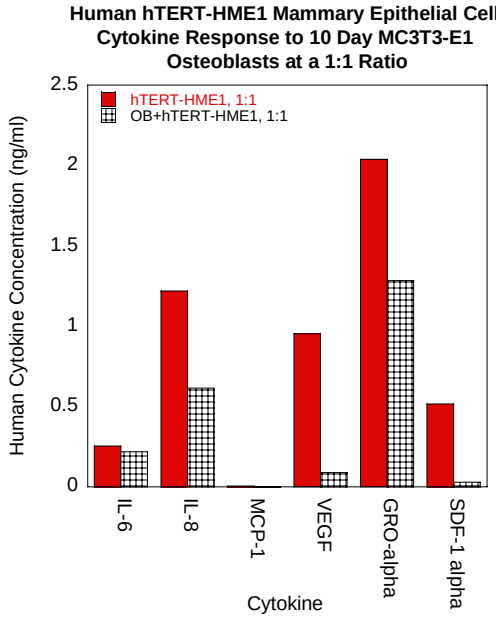
W.


Human MDA-MB-468P Non-metastatic Breast Cancer Cell Cytokine Response to 20 Day Osteoblasts at a 1:1 Ratio
MDA-468P, 1:1
OB+MDA-468P, 1:1
Human Cytokine Concentration (ng/ml)
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

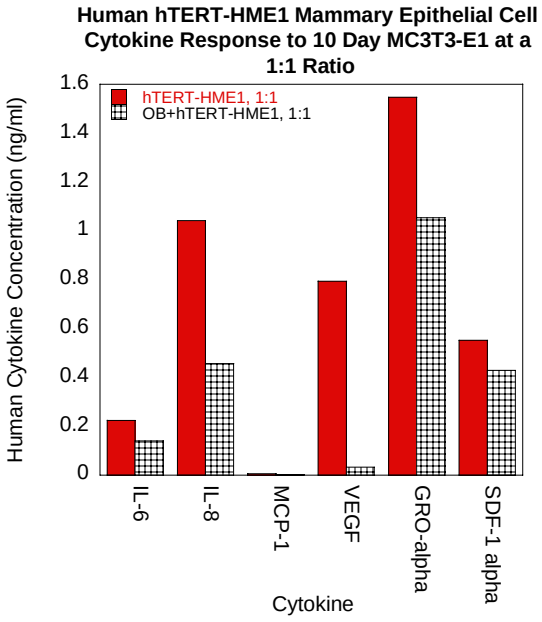
X.


Human MDA-MB-468P Non-metastatic Breast Cancer Cell Cytokine Response to 20 Day MC3T3-E1 Osteoblasts at a 1:1 Ratio
MDA-468P, 1:1
OB+MDA-468P, 1:1
Human Cytokine Concentration (ng/ml)
2
1.5
1
0.5
0
IL-6
IL-8
MCP-1
VEGF
GRO-alpha
SDF-1 alpha
Cytokine

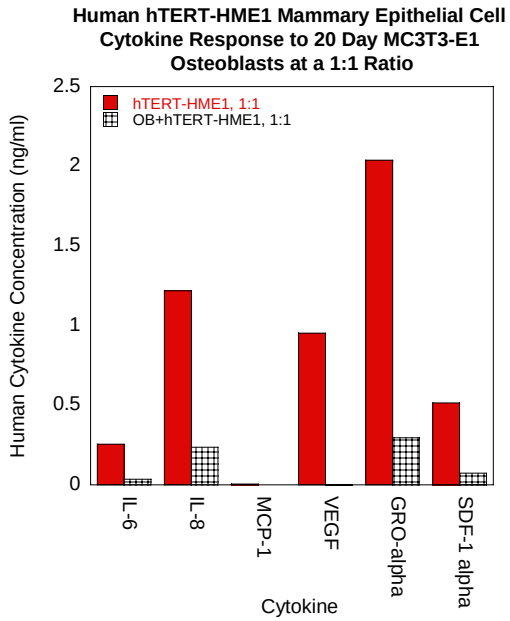
Y.



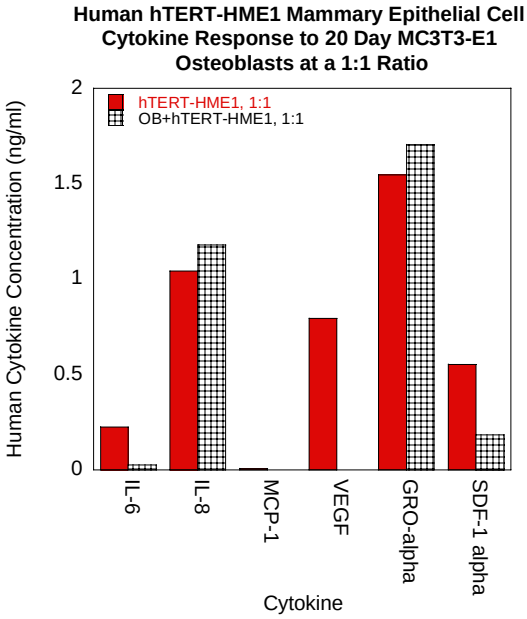
Z.



A2.



B2.



Human transwell cell variants were plated in the insert of a transwell system at ratios of 1:1 by themselves or with 10 (A-B, E-F, I-J, M-N, Q-R, U-V, Y-Z) or 20 (C-D, G-H, K-L, O-P, S-T, W-X, A2-B2) day old MC3T3-E1 osteoblasts and incubated for an additional 3 days. Breast Cancer Cell-derived Human cytokine concentrations were

quantified using a human Bio-Rad Bio-Plex (10 Day old osteoblasts: A. Set A MDA-231W, B. Set B MDA-231W, E. Set A MDA-231PY, F. Set B MDA-231PY, I. Set A MDA-231BO, J. Set B MDA-231BO, M. Set A MDA-231BR, N. Set B. MDA-231BR; Q. Set A MDA-231BRMS, R. Set B MDA-231BRMS, U. Set A MDA-468P, V. Set B MDA-468P, Y. Set A hTERT-HME1, Z. Set B hTERT-HME1. 20 Day old osteoblasts: C. Set A MDA-231W, D. Set B MDA-231W, G. Set A MDA-231PY, H. Set B MDA-231PY, K. Set A MDA-231BO, L. Set B MDA-231BO, O. Set A MDA-231BR, P. Set B. MDA-231BR; S. Set A MDA-231BRMS, T. Set B MDA-231BRMS, W. Set A MDA-468P, X. Set B MDA-468P, A2. Set A hTERT-HME1, B2. Set B hTERT-HME1 .).

Figure 20: Illustration of Experimental Set-up for Mice Intracardiac Inoculations

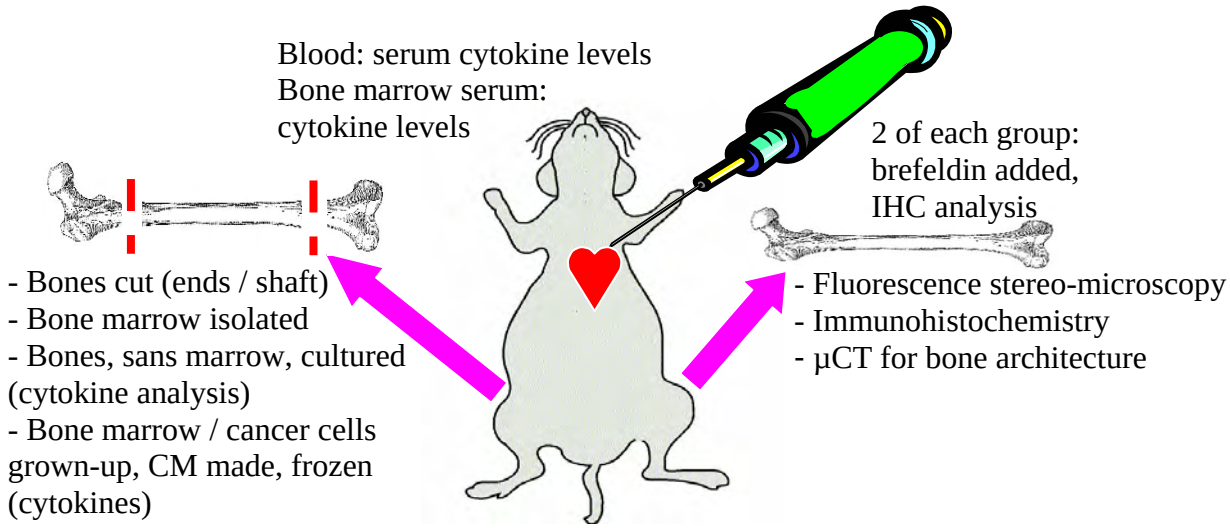
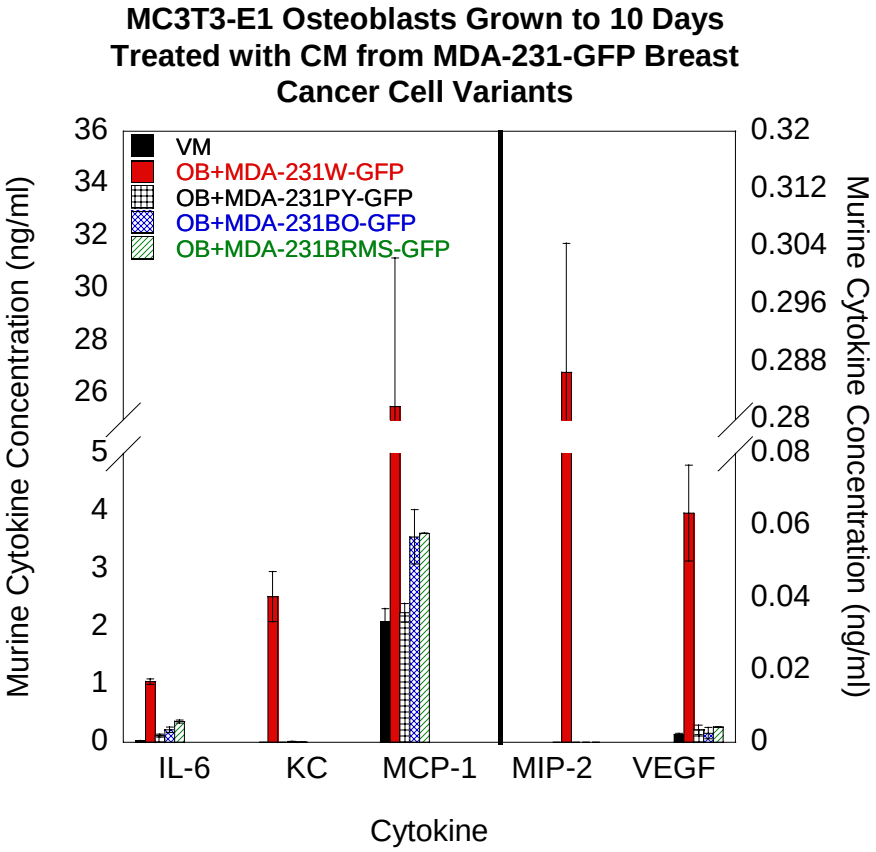


Figure 21.: Murine MC3T3-E1 cytokine response to osteoblasts grown to 10 days old and treated with 0 (VM) or 50% conditioned medium from human MDA-231-GFP breast cancer cell variants.



Murine MC3T3-E1 osteoblasts grown to 10 days were incubated with 0 or 50% conditioned medium from MDA-231-GFP breast cancer cell variants for 24 hrs. Cytokines in the medium were quantified using Bio-Rad Bio-Plex™ Murine Cytokine quantification array.

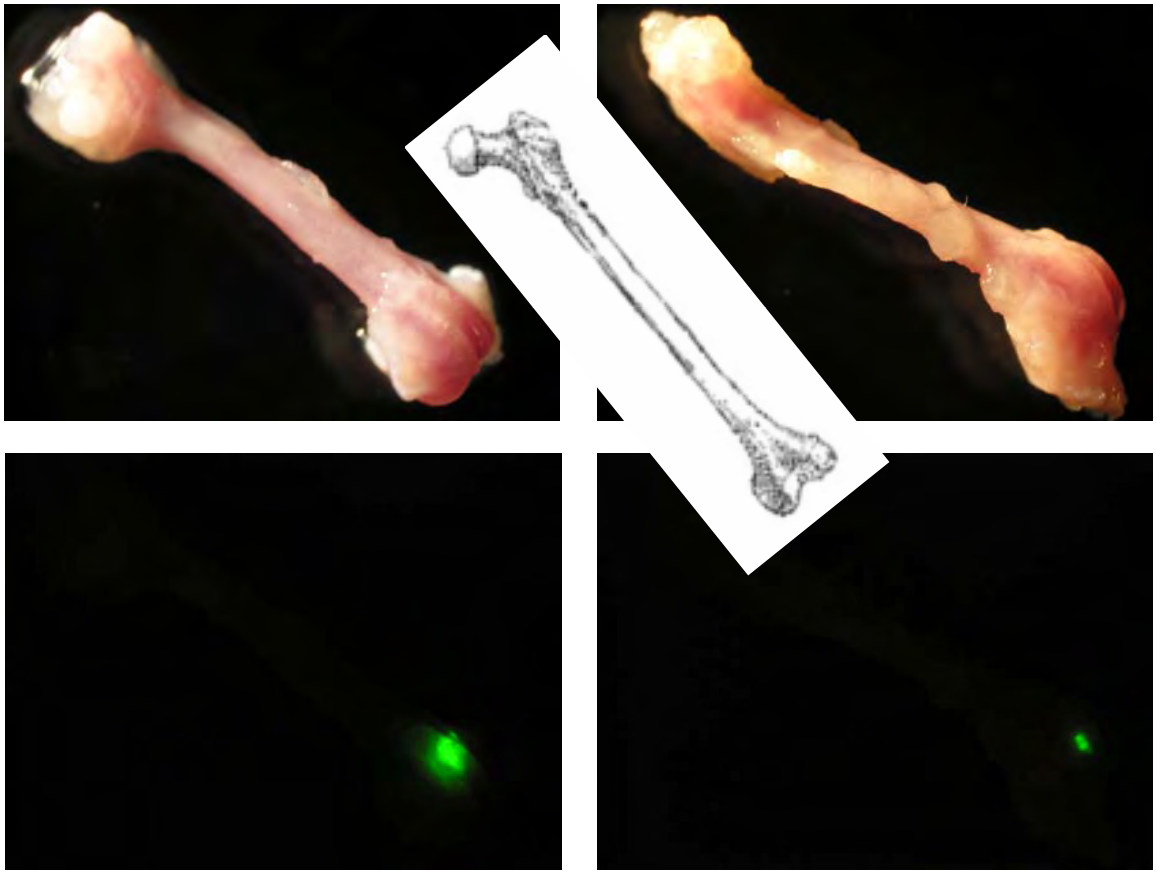
Figure 22: Photograph of a murine femur from a mouse treated with Brefeldin A versus a murine femur from a mouse not treated with Brefeldin A.

Bones from Mice Injected with Brefeldin A

Bones from Mice NOT Injected with Brefeldin A

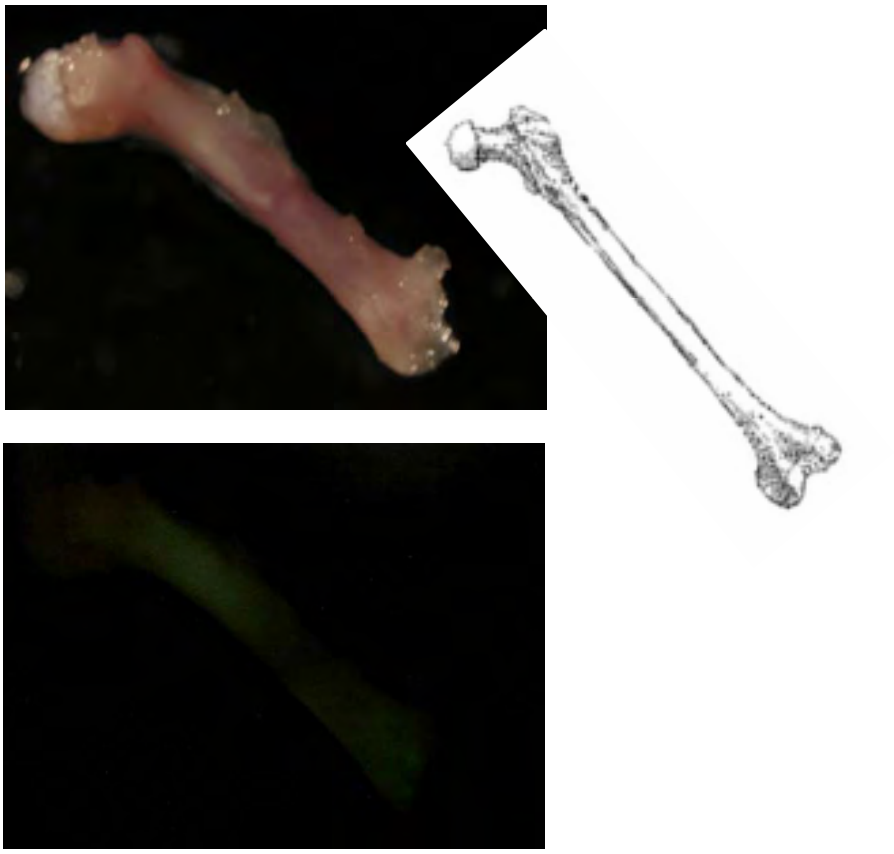
Figure 23: Fluorescence microscopy images of murine femurs bearing visible tumors.

A. MDA-231W GFP



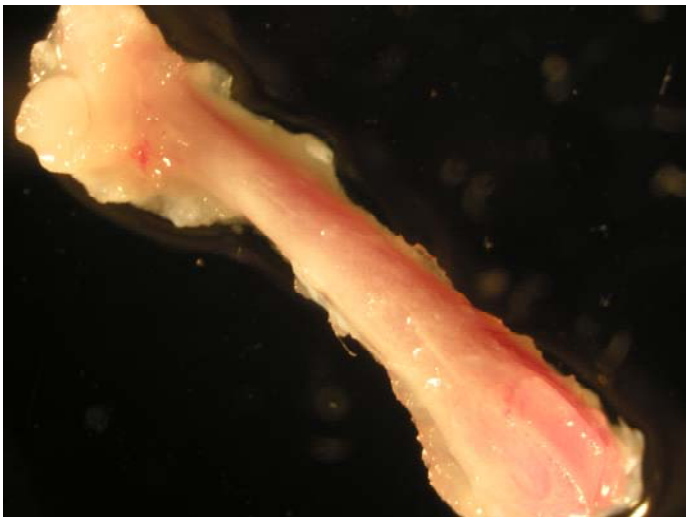
B.

MDA-231PY GFP

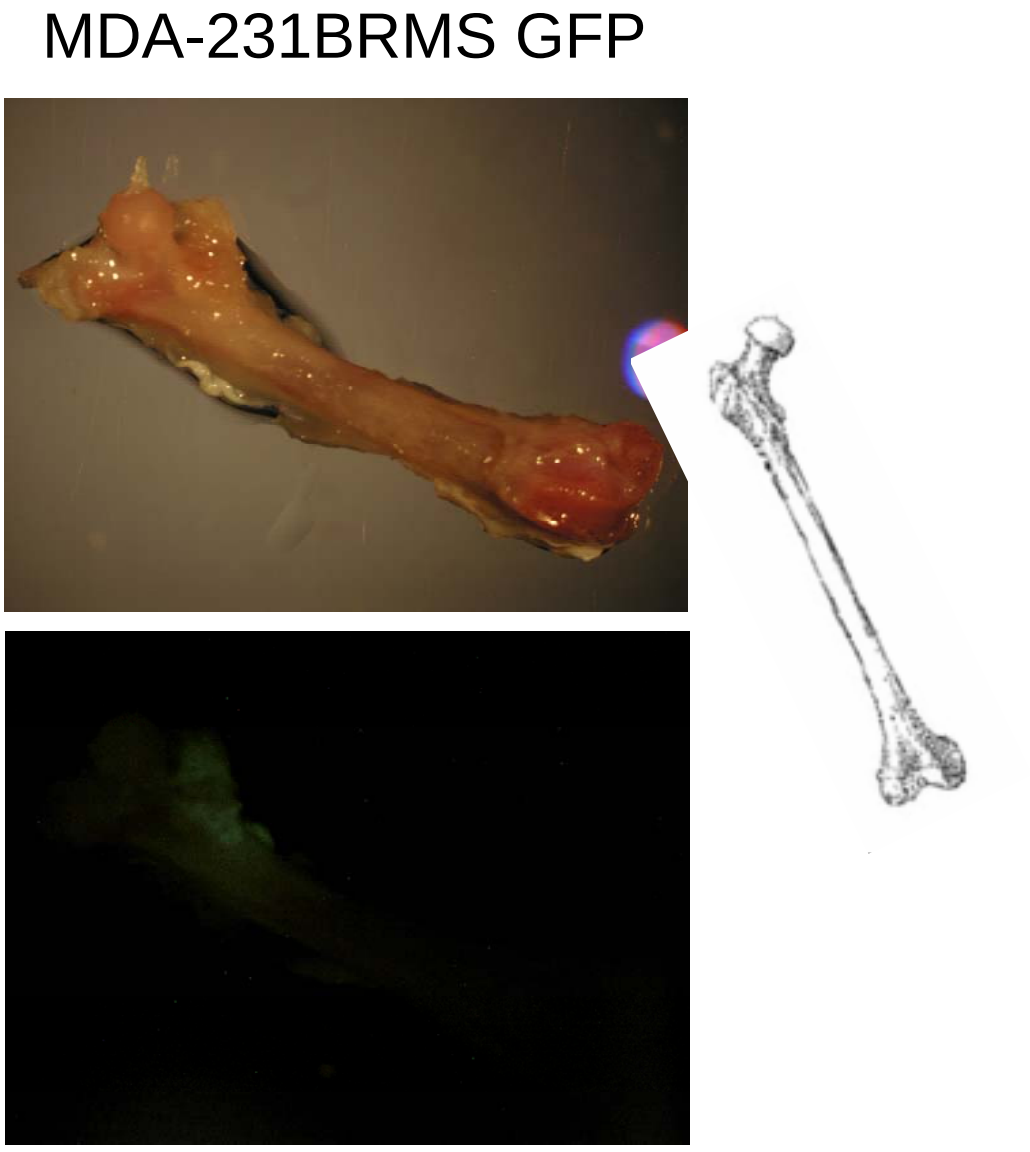


C.

MDA-231BO GFP



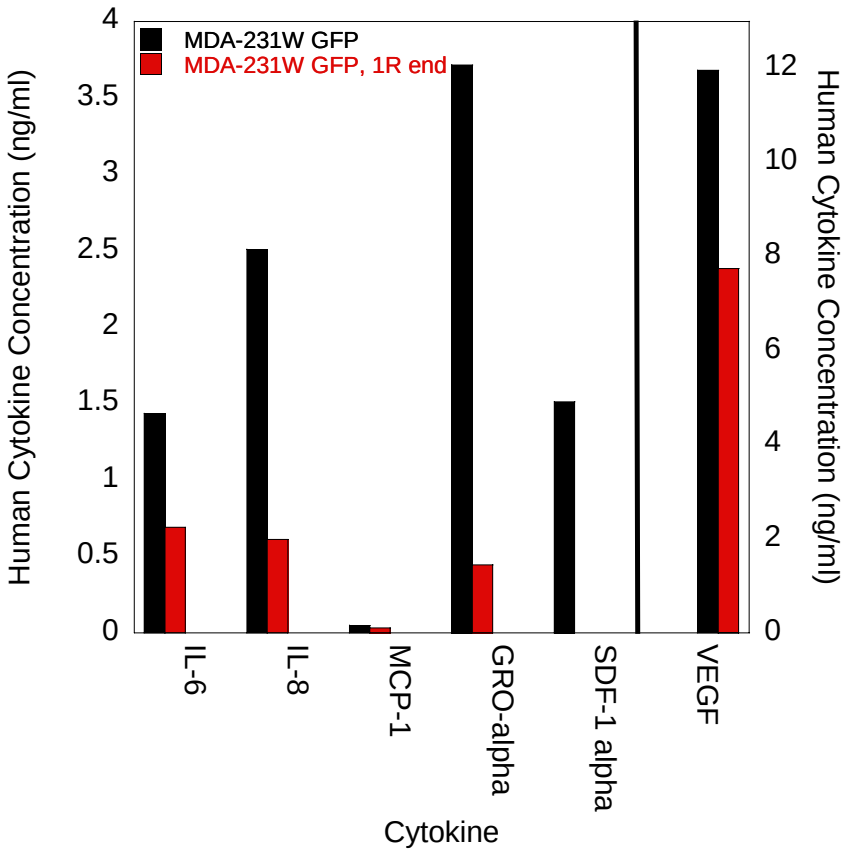
D.



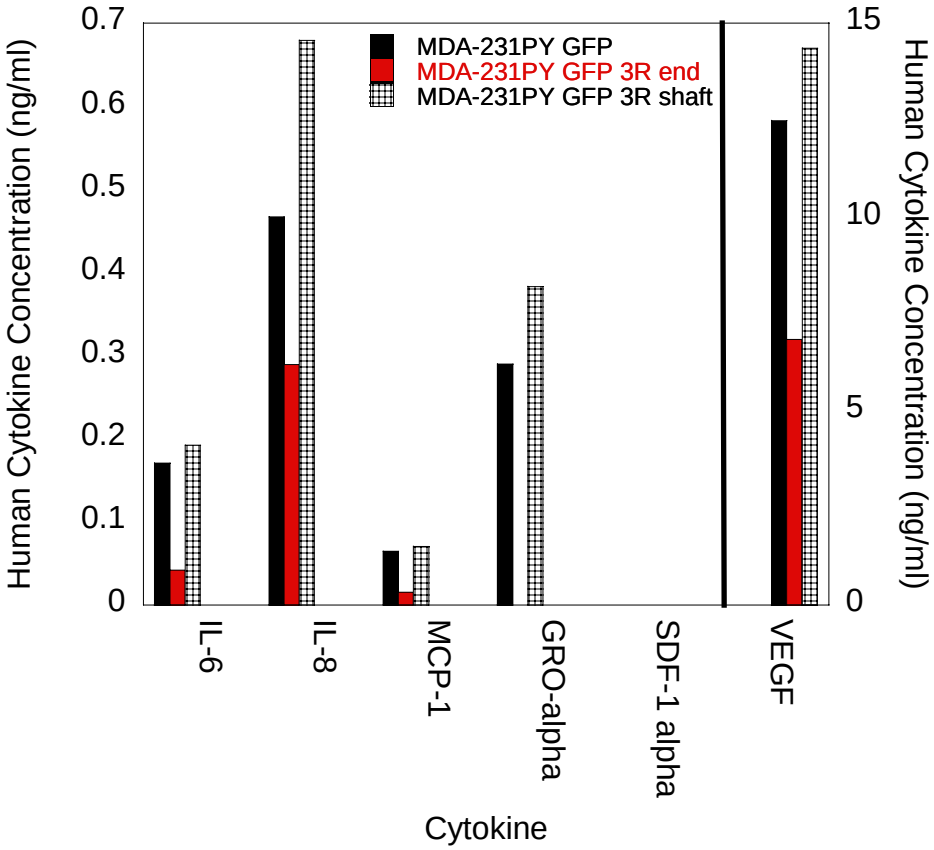
MDA-MB-231-GFP cell variants (3 x 10^5 cells) were inoculated into the left cardiac ventricle of 4-6 week old athymic, female mice. Control mice were untreated. Mice were euthanized at 3 weeks and femurs harvested. Murine femurs were photographed using a fluorescent microscope.

Figure 24.: The Human Cytokine Expression of Retrieved Intracardiac Inoculated Human Metastatic Breast Cancer Cell GFP Variants.

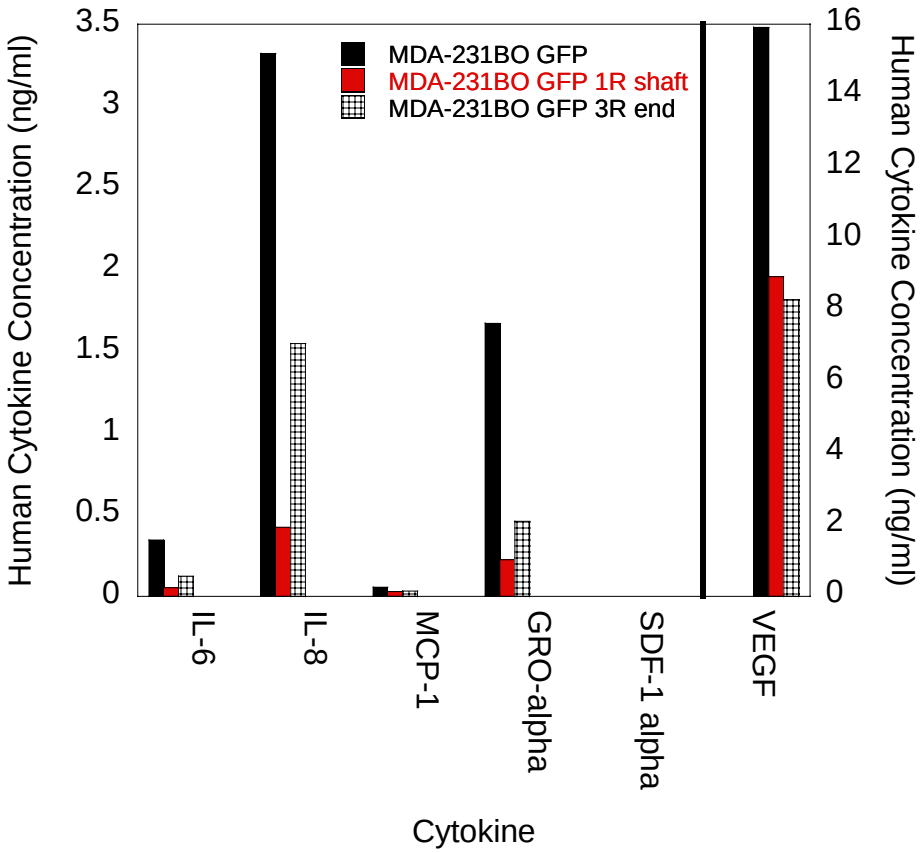
A.



B.



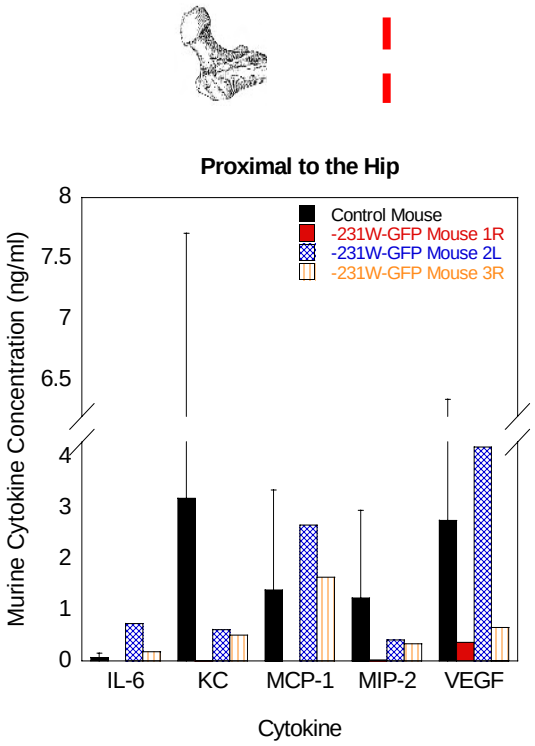
C.



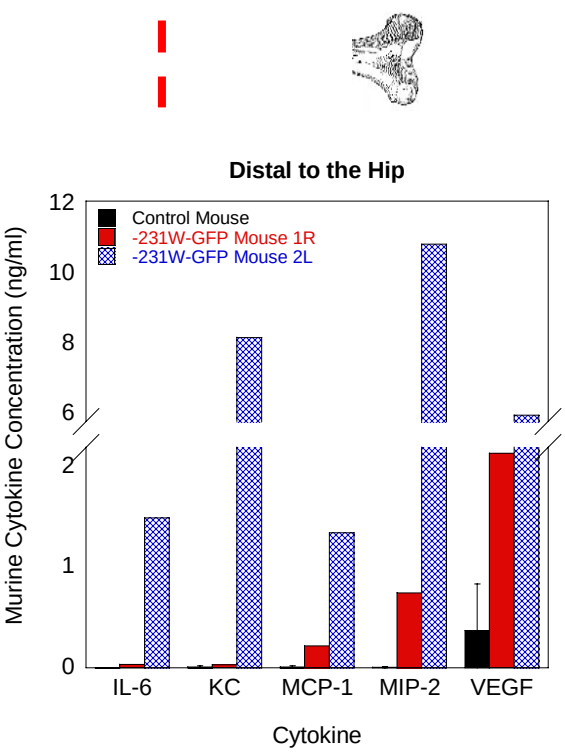
Human metastatic breast cancer cell GFP variants were retrieved from the femoral bone marrow of intracardiac inoculated mice. Cells were expanded from 24-well plates into tissue culture flasks and CM was prepared. Human cytokines in the medium were quantified using Bio-Rad Bio-Plex™ Human Cytokine quantification array. A) Retrieved MDA-MB-231W-GFP variants, B) retrieved MDA-MB-231PY-GFP variants, C) retrieved MDA-MB-231BO-GFP variants.

Figure 25: Cytokine expression of murine femur metaphyses and diaphyses ex-vivo following intracardiac inoculation.

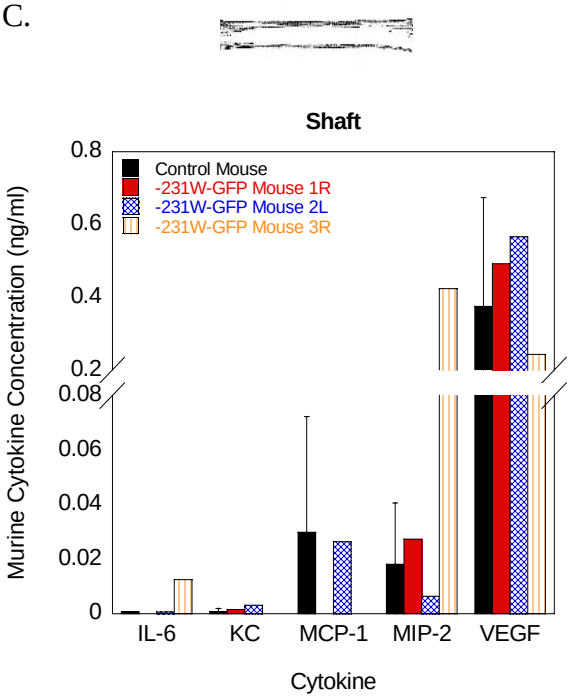
A.



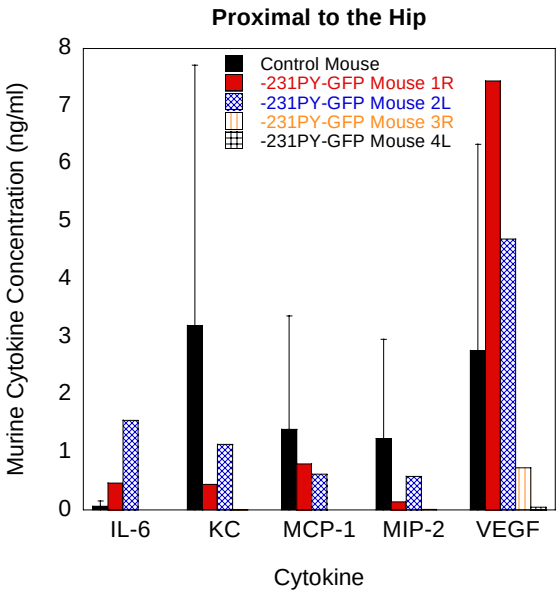
B.



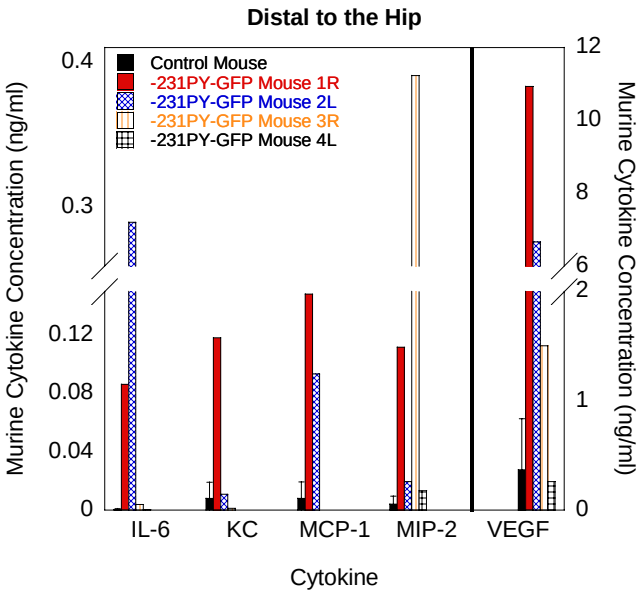
C.



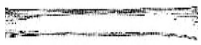
D.

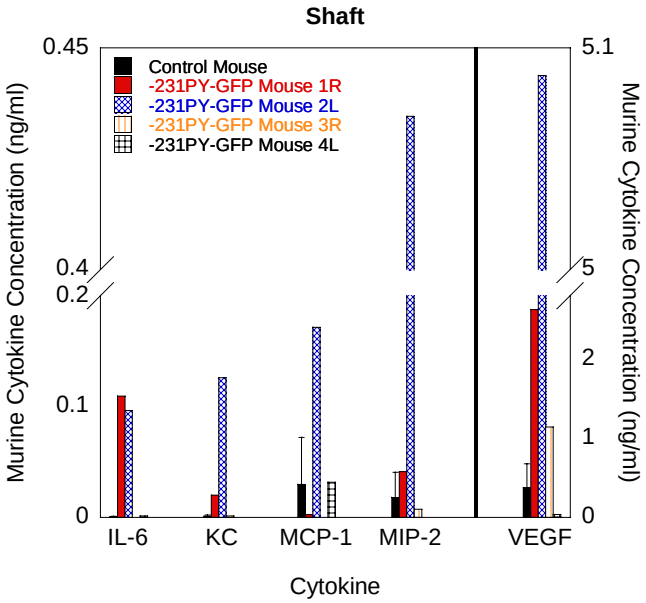

Proximal to the Hip
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231PY-GFP Mouse 1R
-231PY-GFP Mouse 2L
-231PY-GFP Mouse 3R
-231PY-GFP Mouse 4L
0
1
2
3
4
5
6
7
8
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

E.

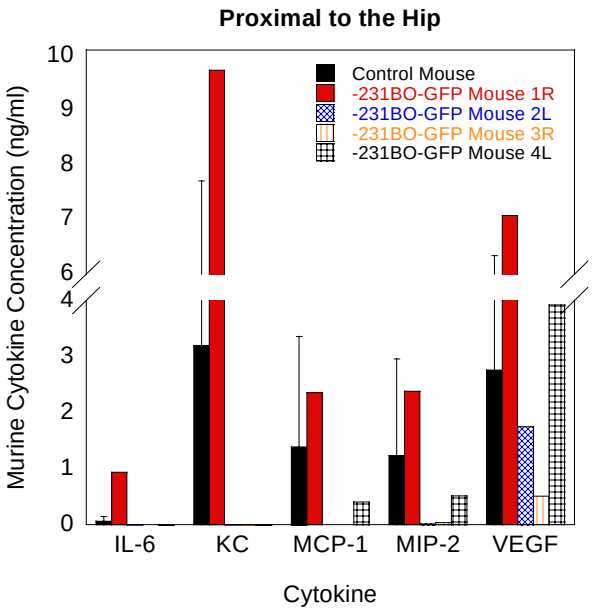

Distal to the Hip
Murine Cytokine Concentration (ng/ml)
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231PY-GFP Mouse 1R
-231PY-GFP Mouse 2L
-231PY-GFP Mouse 3R
-231PY-GFP Mouse 4L
0
0.04
0.08
0.12
0.3
0.4
0
1
2
6
8
10
12
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

F.

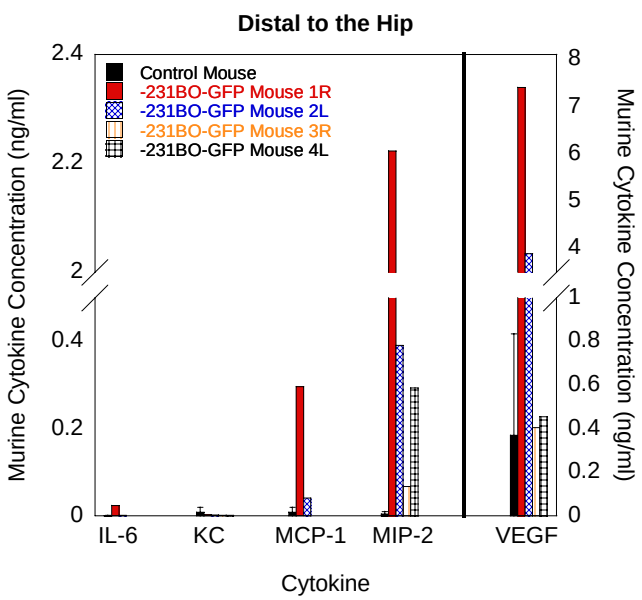



Shaft
Murine Cytokine Concentration (ng/ml)
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231PY-GFP Mouse 1R
-231PY-GFP Mouse 2L
-231PY-GFP Mouse 3R
-231PY-GFP Mouse 4L
0
0.1
0.2
0.4
0.45
0
1
2
5
5.1
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

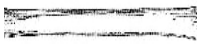
G.

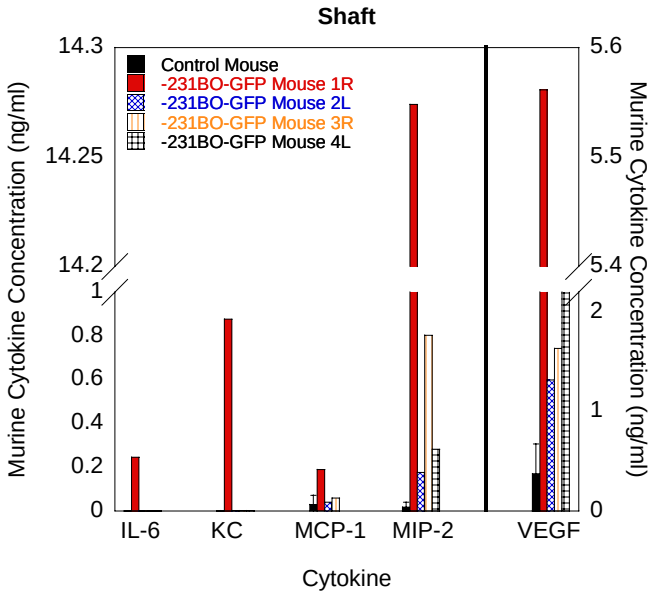

Proximal to the Hip
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231BO-GFP Mouse 1R
-231BO-GFP Mouse 2L
-231BO-GFP Mouse 3R
-231BO-GFP Mouse 4L
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

H.

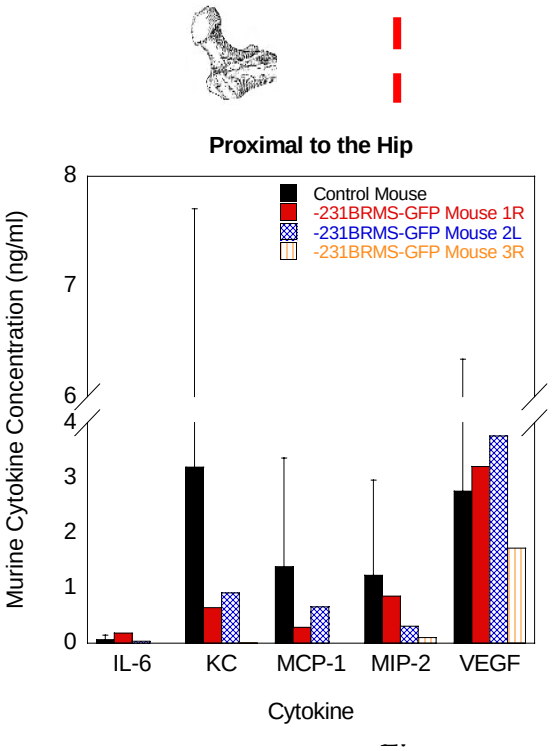

Distal to the Hip
Murine Cytokine Concentration (ng/ml)
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231BO-GFP Mouse 1R
-231BO-GFP Mouse 2L
-231BO-GFP Mouse 3R
-231BO-GFP Mouse 4L
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

I.

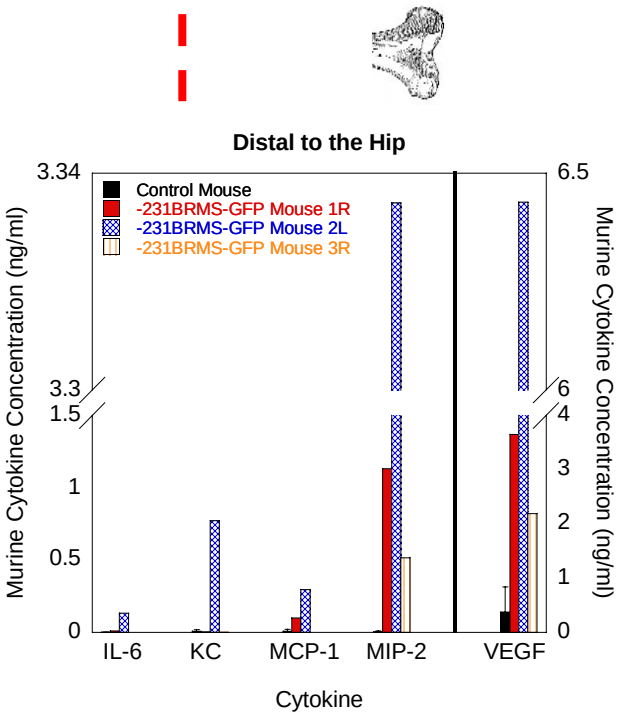



Shaft
Murine Cytokine Concentration (ng/ml)
Murine Cytokine Concentration (ng/ml)
Control Mouse
-231BO-GFP Mouse 1R
-231BO-GFP Mouse 2L
-231BO-GFP Mouse 3R
-231BO-GFP Mouse 4L
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

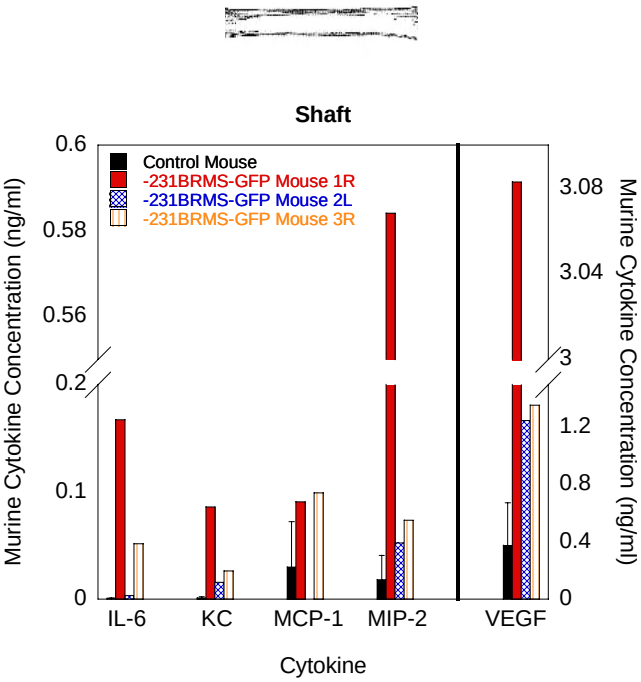
J.


Proximal to the Hip
Control Mouse
-231BRMS-GFP Mouse 1R
-231BRMS-GFP Mouse 2L
-231BRMS-GFP Mouse 3R
Murine Cytokine Concentration (ng/ml)
8
7
6
4
3
2
1
0
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

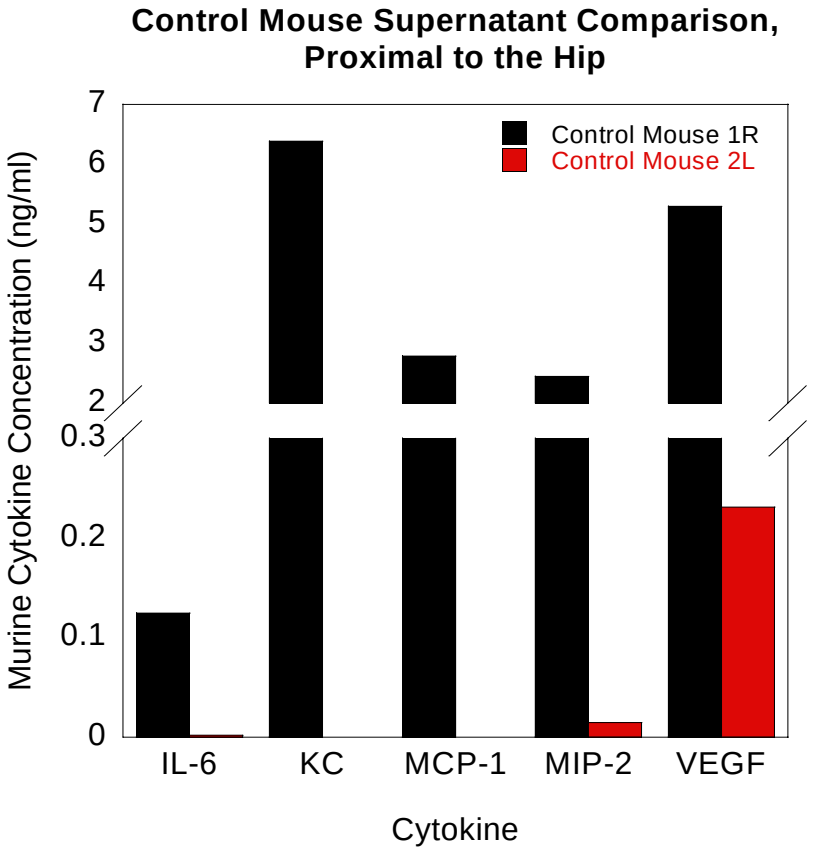
K.


Distal to the Hip
Control Mouse
-231BRMS-GFP Mouse 1R
-231BRMS-GFP Mouse 2L
-231BRMS-GFP Mouse 3R
Murine Cytokine Concentration (ng/ml)
3.34
3.3
1.5
1
0.5
0
6.5
6
4
3
2
1
0
Murine Cytokine Concentration (ng/ml)
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

L.


Shaft
Control Mouse
-231BRMS-GFP Mouse 1R
-231BRMS-GFP Mouse 2L
-231BRMS-GFP Mouse 3R
Murine Cytokine Concentration (ng/ml)
0.6
0.58
0.56
0.2
0.1
0
3.08
3.04
3
1.2
0.8
0.4
0
Murine Cytokine Concentration (ng/ml)
IL-6
KC
MCP-1
MIP-2
VEGF
Cytokine

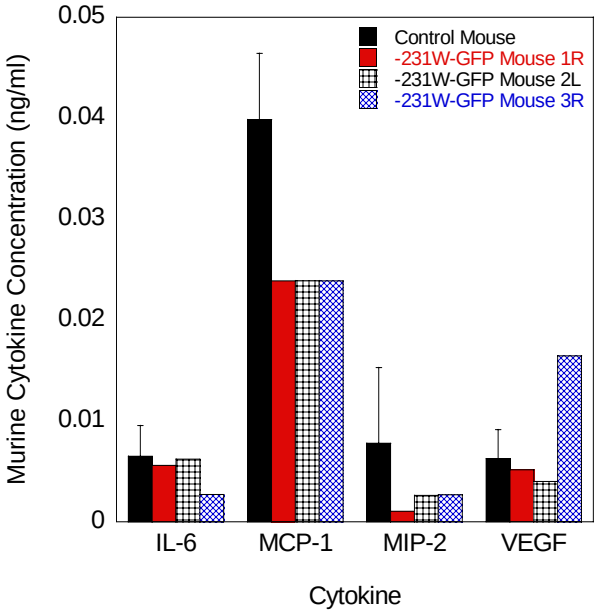
M.



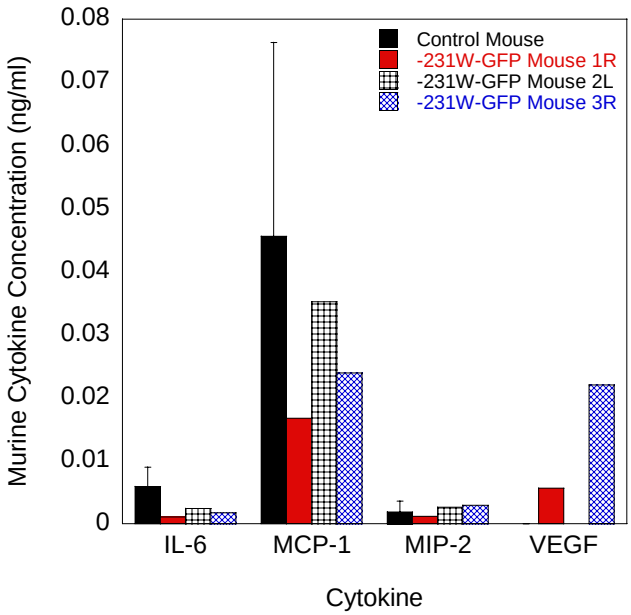
MDA-MB-231-GFP cell variants (3 x 10^5 cells) were inoculated into the left cardiac ventricle of 4-6 week old athymic, female mice. Control mice were untreated. Mice were euthanized at 3 weeks and femurs harvested. Femur metaphyses were fractionated. Isolated metasphyseal bone pieces were crushed and cultured. Media were collected and tested after 24 hours. Murine IL-6, MIP-2, KC, MCP-1, and VEGF were quantified using a Bio-Rad Bio-Plex™ murine cytokine quantification assay.

Figure 26: Cytokine expression of murine femur metaphyses and diaphysis bone marrow serum ex-vivo following intracardiac inoculation.

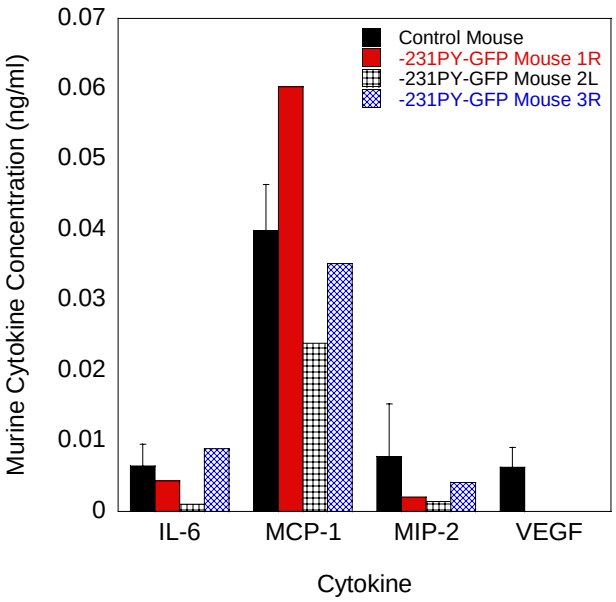
A.: Ends



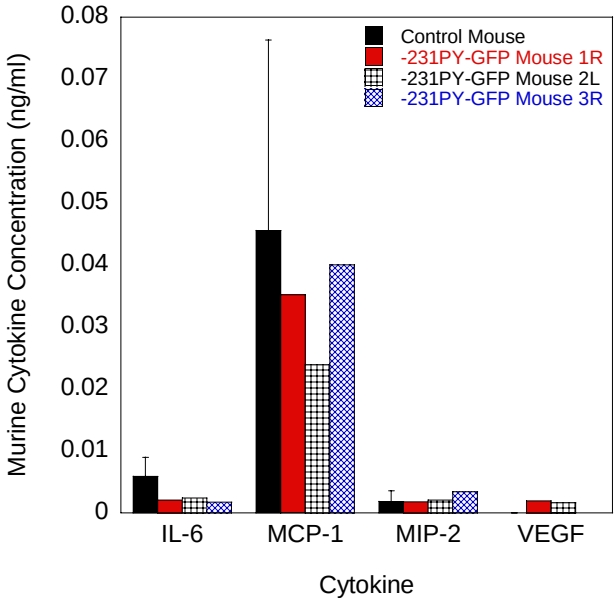
B.: Shaft



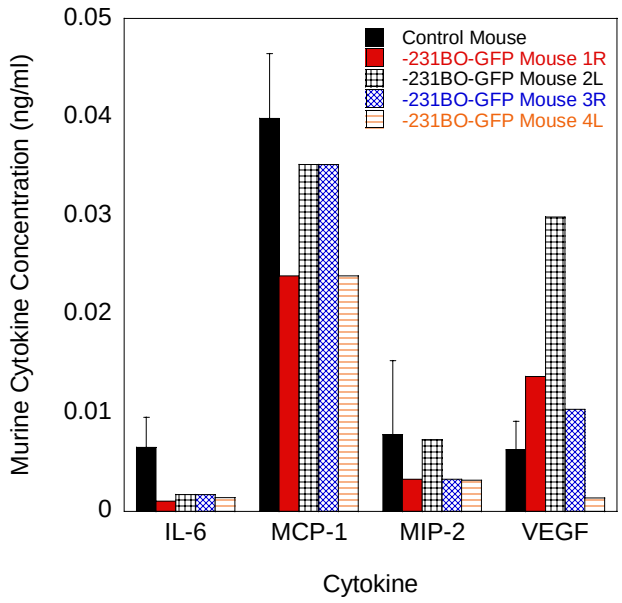
C.: Ends



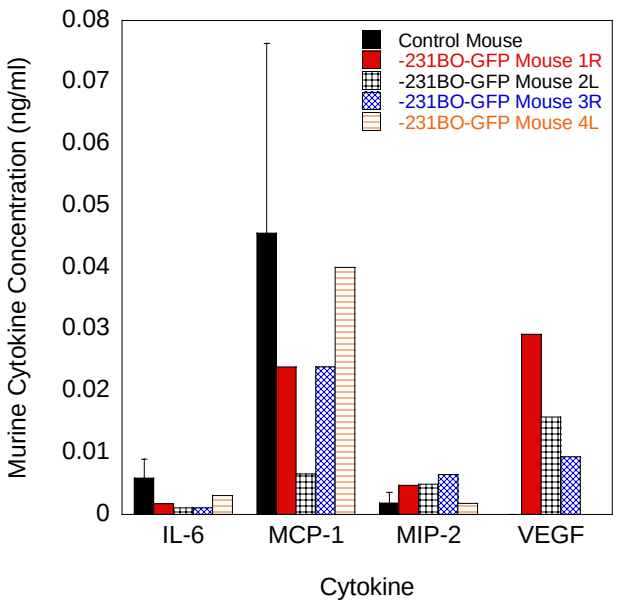
D.: Shaft



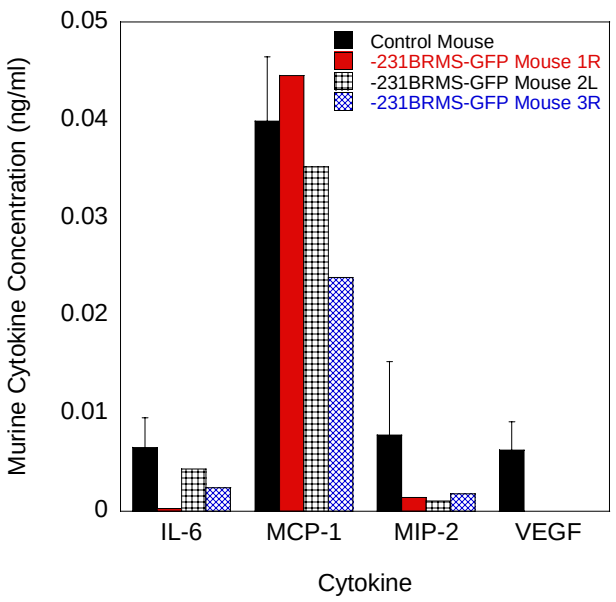
E.: Ends



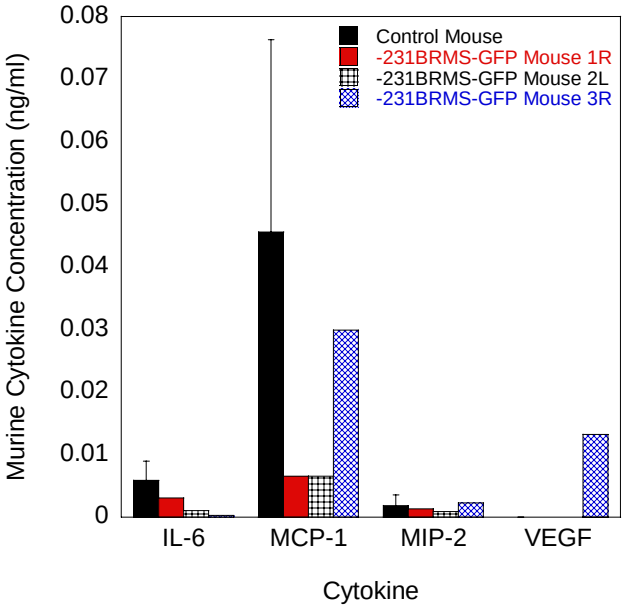
F.: Shaft



G.: Ends



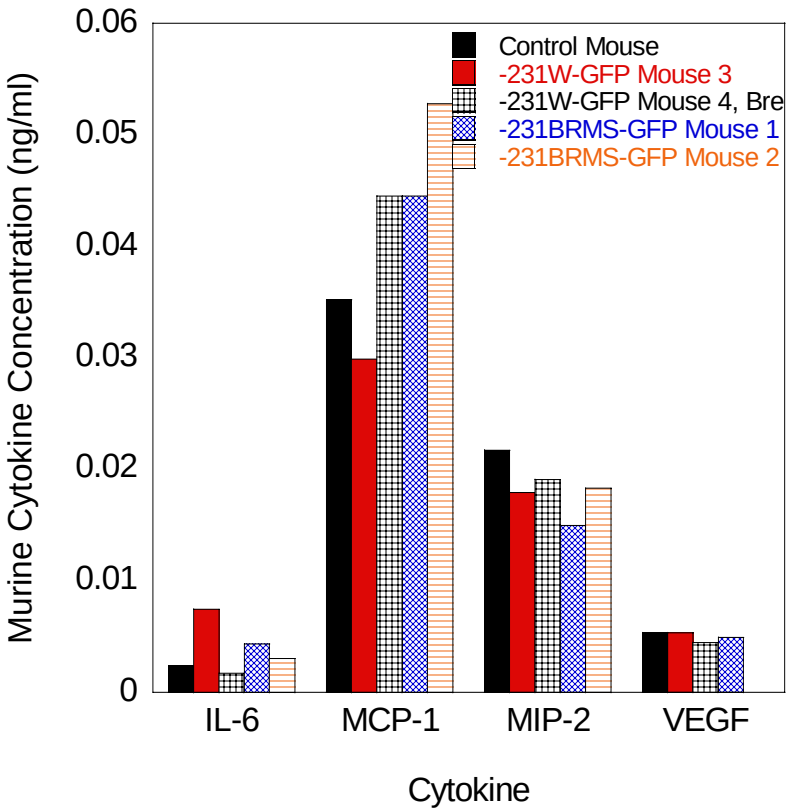
H.: Shaft



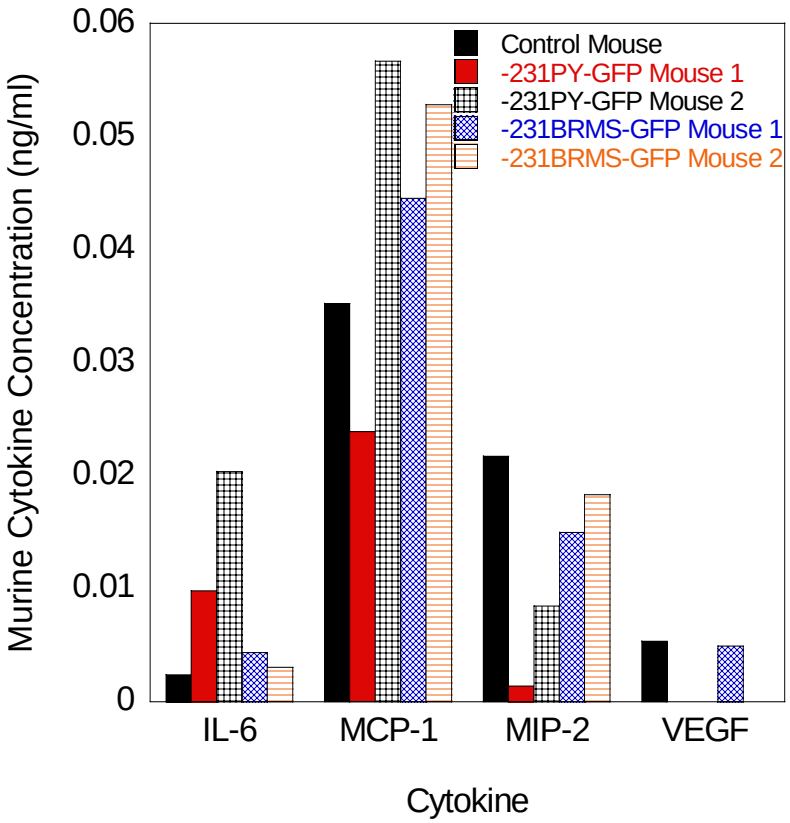
MDA-MB-231-GFP cell variants (3×10^5 cells) were inoculated into the left cardiac ventricle of 4-6 week old athymic, female mice. Control mice were untreated. Mice were euthanized at 3 weeks and femurs harvested. Femur metaphyses were fractionated. Isolated metasphyseal and diaphysis bone pieces were flushed no less than 5 times each with 1 ml of PBS. Bone marrow serum was extracted by centrifugation of the red cells, and then refrigeration overnight. The top serum fraction was collected and for murine cytokine expression of IL-6, MIP-2, KC, MCP-1, and VEGF using a Bio-Rad Bio-Plex™ murine cytokine quantification assay. A) Bone marrow serum cytokine expression from the ends of MDA-231W-GFP mice, B) bone marrow serum cytokine expression from the shaft of MDA-231W-GFP mice, C) bone marrow serum cytokine expression from the ends of MDA-231PY-GFP mice, D) bone marrow serum cytokine expression from the shaft of MDA-231PY-GFP mice, E) bone marrow serum cytokine expression from the ends of MDA-231BO-GFP mice, F) bone marrow serum cytokine expression from the shaft of MDA-231BO-GFP mice, G) bone marrow serum cytokine expression from the ends of MDA-231BRMS-GFP mice, H) bone marrow serum cytokine expression from the shaft of MDA-231BRMS-GFP mice.

Figure 27: Cytokine expression of murine blood serum ex-vivo following intracardiac inoculation

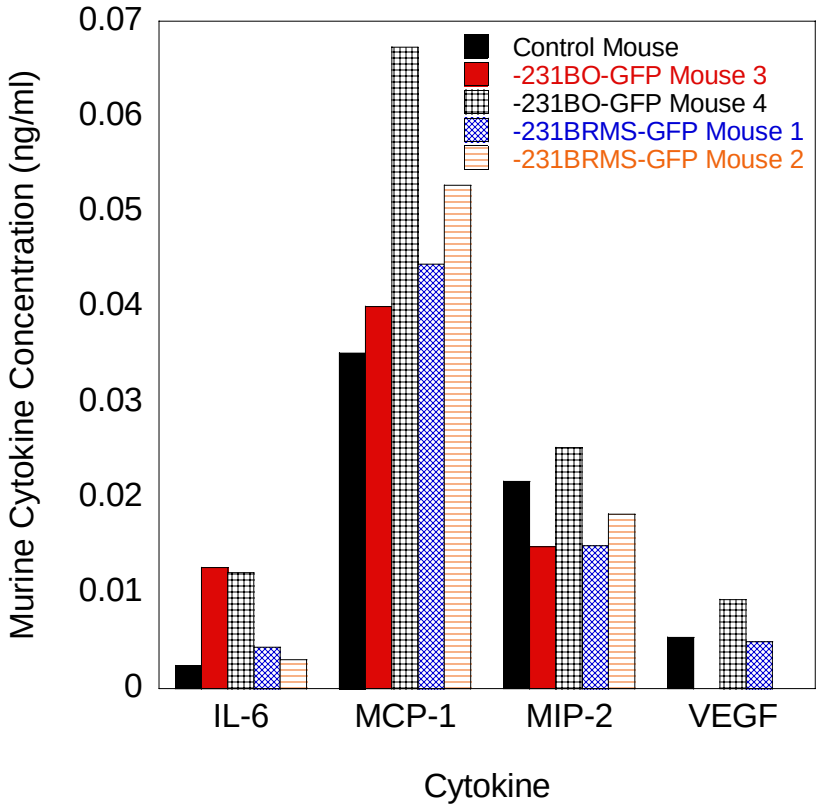
A.



B.



C.



MDA-MB-231-GFP cell variants (3 x 10^5 cells) were inoculated into the left cardiac ventricle of 4-6 week old athymic, female mice. Control mice were untreated. Mice were euthanized at 3 weeks. Murine blood was extracted by removing the eye from the orbital socket and gently massaging the inverted, freshly euthanized carcass from tail to head to obtain fresh blood. Blood was refrigerated overnight to obtain serum. The top serum fraction was collected and assayed for murine cytokine expression of IL-6, MIP-2, KC, MCP-1, and VEGF using a Bio-Rad Bio-Plex™ murine cytokine quantification assay. A) Blood serum cytokine expression of control, MDA-MB-231W-GFP inoculated mice, and MDA-MB-231BRMS-GFP inoculated mice, B) blood serum cytokine expression of control, MDA-MB-231PY-GFP inoculated mice, and MDA-MB-231BRMS-GFP inoculated mice, and C) blood serum cytokine expression of control, MDA-MB-231BO-GFP inoculated mice, and MDA-MB-231BRMS-GFP inoculated mice.

Figure 28: Micro-CT scan of the metaphysis of a non-cancer-bearing murine femur.

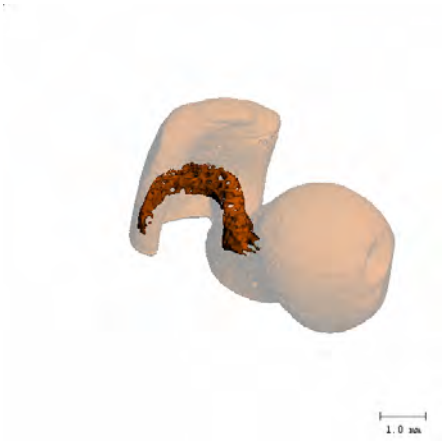


Table 2: Known cytokine receptors.

	MDA-231	**MC3T3-E1**	**Osteoclast**
IL-6R (IL-6)	+/- (?) Protein (in-situ samples)	+	!
CXCR1/2 (IL-8/MIP-2)	Protein (flow) invasion	unknown	!
CXCR1/2 (GRO-α/KC)	Protein (flow) invasion	unknown	+
VEGFR1/Flt VEGFR2/KDR VEGFR3/NRP1 (VEGF)	Protein (IP & WB) (all)	+/- (?)	!
CCR2 (MCP-1)	Protein (flow) invasion	+	+

Figure 29: Illustration of Experimental Set-up for Breast Cancer Migration Assay (Chemoattraction)

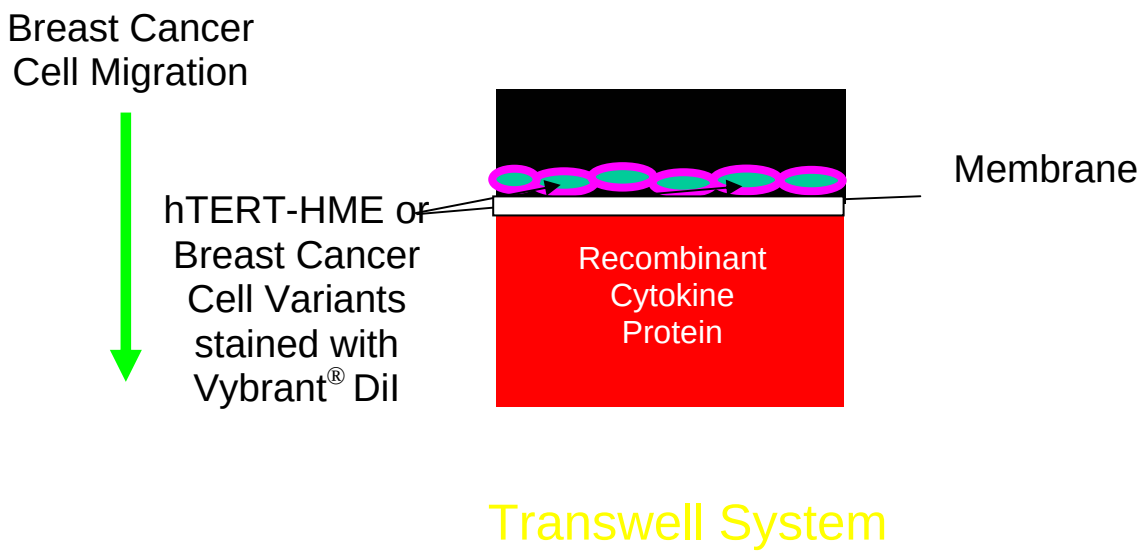
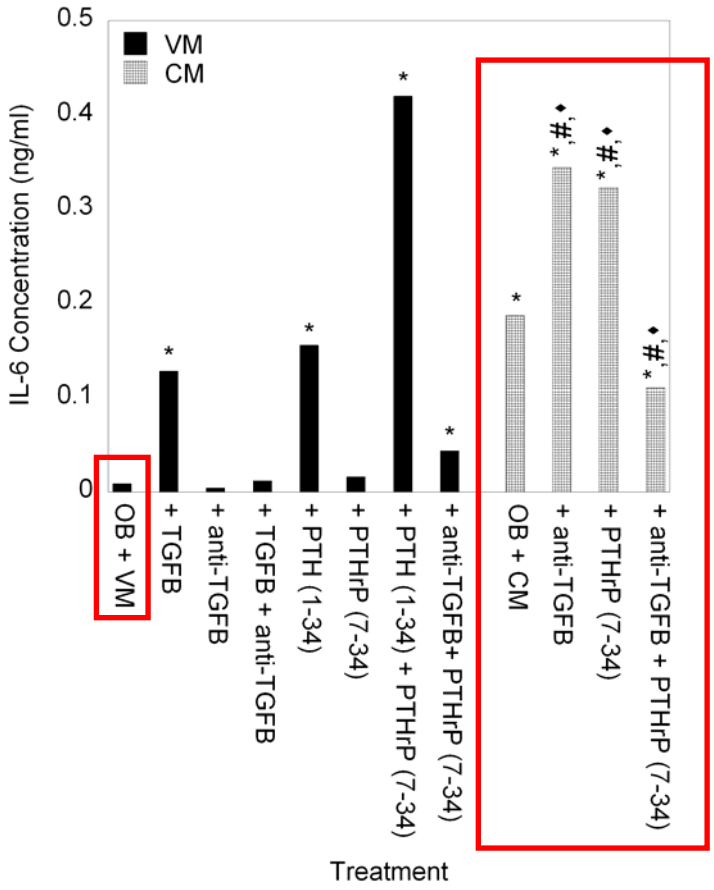


Figure 30: IL-6 Concentration is Not Reduced to VM Levels By anti-TGFB or PTHrP (7-34).



Murine MC3T3-E1 osteoblasts grown to 16 days were treated for 24 hours with VM, 50% MDA-231W CM, TGF-β, anti- TGF-β, PTH, or PTHrP in a variety of different scenerios. Cytokines in the medium were quantified using Bio-Rad Bio-Plex™ Murine Cytokine quantification array.

APPENDICES

1) Publication: 2008 Kinder, M, Chislock, EM, **Bussard, KM**, Shuman, LA, Mastro, AM. Metastatic Breast Cancer Induces an Osteoblast Inflammatory Response. Experimental Cell Research. 314: (1), 173.

2) Publication: 2008 **Bussard, KM**, Gay, CV, Mastro, AM. The Microenvironment in Metastasis: What is Special About Bone? Accepted to Cancer Metastasis Reviews. Available on Epub ahead of print December 11, 2007.

3) Abstract: 2007 **Bussard, KM**, Mastro, AM. "Osteoblasts Naturally Produce Cytokines that Influence the Tumor Microenvironment in Bone Metastatic Breast Cancer." Skeletal Complications of Malignancy V, The Paget Foundation. October 25-27, 2007.

4) Abstract: 2008 **Bussard, KM**, Mastro, AM. "Osteoblast-Derived Cytokines are Major Mediators in Facilitating Bone Metastatic Breast Cancer." Presented at the American Association for Cancer Research Annual Meeting's Tumor Biology Minisymposium, April 12-16, 2008.

5) Bussard, KM. Curriculum vitae.

EXPERIMENTAL CELL RESEARCH 314 (2008) 173–183

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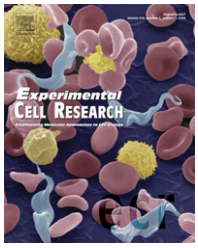

Research Article

Metastatic breast cancer induces an osteoblast inflammatory response

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ABSTRACT

Breast cancer preferentially metastasizes to the skeleton, a hospitable environment that attracts and allows breast cancer cells to thrive. Growth factors released as bone is degraded support tumor cell growth, and establish a cycle favoring continued bone degradation. While the osteoclasts are the direct effectors of bone degradation, we found that osteoblasts also contribute to bone loss. Osteoblasts are more than intermediaries between tumor cells and osteoclasts. We have presented evidence that osteoblasts contribute through loss of function induced by metastatic breast cancer cells. Metastatic breast cancer cells suppress osteoblast differentiation, alter morphology, and increase apoptosis. In this study we show that osteoblasts undergo an inflammatory stress response in the presence of human metastatic breast cancer cells. When conditioned medium from cancer cells was added to human osteoblasts, the osteoblasts were induced to express increased levels of IL-6, IL-8, and MCP-1; cytokines known to attract, differentiate, and activate osteoclasts. Similar findings were seen with murine osteoblasts and primary murine calvarial osteoblasts. Osteoblasts are co-opted into creating a microenvironment that exacerbates bone loss and are prevented from producing matrix proteins for mineralization. This is the first study implicating osteoblast produced IL-6, IL-8 (human; MIP-2 and KC mouse), and MCP-1 as key mediators in the osteoblast response to metastatic breast cancer cells.

Introduction

Breast cancer is the second deadliest form of cancer for women in the United States, largely due to its tendency to metastasize. Once metastasis occurs, the relative 5-year survival rate drops precipitously from over 90% to less than 10% depending on the site of the metastasis. For breast cancer, the skeleton is the preferred site of metastasis. Nearly 50% of primary and about 70% of secondary metastases target bone [1–3]. Within the skeletal system, breast cancer cells most frequently colonize the ends of long bones, ribs, and vertebrae; these areas contain rich microvasculature closely juxtaposed to metabolically active trabecular bone surfaces [2].

The metaphyseal area at the ends of long bones contains a complex network of bone cells, hematopoietic cells, and stromal cells. The entry of breast cancer cells into the marrow cavity disturbs the status quo, in particular, the interaction between osteoblasts and osteoclasts. In the adult skeleton,

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these two cell types are responsible for the slow and continuous turnover of bone [4]. When metastatic breast cancer cells invade the bone microenvironment, the balance is upset in favor of net bone loss. Indeed, breast cancer metastasis usually results in osteolytic lesions due to activated osteoclasts that degrade bone matrix. The bone loss can cause severe pain, pathologic fractures, spinal cord and nerve compression, hypercalcemia, and bone marrow suppression [2]). In addition, growth factors released from the matrix promote cancer cell proliferation and contribute to what has been described as "the vicious cycle"[5]. In particular, transforming growth factor-beta (TGF-β) and insulin growth factor-1 (IGF-1), which are released from the matrix during bone degradation, stimulate the production of parathyroid hormone-related protein (PTHrP) that fosters cancer cell growth [5–8].

Furthermore, growth factors released from the matrix, substances secreted by the cancer cells and osteoblasts contribute to the metastatic microenvironment. Some osteoblast factors, such as receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotogerin (OPG), are part of the normal osteoblast-osteoclast signaling cross talk. Others, such as interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1), may indicate an osteoblast inflammatory response. It has been known for a long time that chronic inflammation, which occurs as part of the cancer cell's interaction with the stromal environment, supports cancer progression and metastasis [9]. Fibroblasts, endothelial cells, cells of the blood and lymph vasculature, as well as transient cells of the innate and adaptive immune systems all affect cancer cell growth and metastasis. Under ordinary conditions, communication within the stromal network is carried out by cytokines, chemokines, and other peptides. A disruption of homeostasis by trauma, microorganisms, foreign materials, or cancer cells results in drastic changes in the levels and types of cytokines expressed [10]. The stromal environment in metaphyseal bone is no exception. Any circumstance that changes the balance between osteoblasts and osteoclasts may lead to bone loss. For example, osteomyelitis brought about by *M. tuberculosis* or *S. aureus* is associated with uncontrolled inflammation and especially high levels of IL-8, RANTES, and MCP-1 [11]. Titanium transplant-induced bone loss has been traced to an osteoblast stress response with high levels of IL-8, MCP-1, and IL-6 [12]. These cytokines have been shown to attract and activate osteoclasts as well as cells of the immune system, thus perpetuating bone loss [13].

While it is likely that tumor-infiltrating lymphocytes, neutrophils, and macrophages are a potent source of inflammatory molecules, we present evidence in this paper that metastatic breast cancer cells can directly induce osteoblasts to express increased levels of inflammatory stress response molecules, specifically IL-6, IL-8 (macrophage inflammatory protein-2 [MIP-2], KC), and MCP-1. Moreover, the osteoblast response was mediated by soluble factors and occurred independently of direct cancer cell–osteoblast contact. Their ultimate target is the osteoclast.

Current therapies are directed at blocking osteoclast activity. Bisphosphonates such as clodronate, ibandronate, pamidronate, and zoledronic acid are the current standard of care for most metastases to bone. These synthetic analogues of inorganic pyrophosphates inhibit osteoclast activity and slow lesion formation. Although they reduce skeletal-related events, they are not curative. They do not lead to restoration of the bone and do not eliminate the cancer cells [14]. The osteoblasts appear to be functionally paralyzed [15].

We have previously reported that osteoblasts exposed to metastatic breast cancer cells or their conditioned media show an increase in apoptosis, suppression of production of bone matrix proteins, and a change in morphology [16–18]. The results of this study indicate that the osteoblasts also switch into an inflammatory mode in the presence of the breast cancer cells. These osteoblast-produced inflammatory cytokines, different from those made by the cancer cells, can target osteoclast precursors that are effectors of osteolysis. Therefore, osteoblasts contribute to the osteolytic phenotype due to loss of bone deposition functions as well as to increased production of osteoclast activating cytokines.

Materials and methods

Cell lines

Osteoblasts

hFOB 1.19 cells are human fetal osteoblasts that have been immortalized with a temperature-sensitive SV40 large T antigen. At a permissive temperature of 34 °C, they proliferate; incubation at 39 °C show their growth and fosters osteoblast differentiation [19]. These cells, a gift from Dr. Thomas Spelsberg, were cultured at 34 °C, 5% CO_2 in hFOB growth medium, which consists of Dulbecco's Modified Eagle's Medium (DMEM):Ham's F-12 (1:1) (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS) (Sigma), and penicillin 100 U/ml/streptomycin 100 μg/ml (Sigma). For experiments, hFOB 1.19 osteoblasts at 85–90% confluency were cultured at 39 °C, for 2–3 days in a hFOB differentiation medium, [DMEM:Ham's F-12 1:1, 10% charcoal-stripped FBS, penicillin 100 U/ml/streptomycin 100 μg/ml, 10^{-8} M Vitamin D_3 (Biomol, Plymouth Meeting, PA), 10^{-8} M Vitamin K (menadione) (Sigma), and 50 μg/ml Vitamin C (ascorbic acid) (Sigma)]. As indicated for various experiments, hFOB 1.19 cells were plated at approximately 4×10^3 cells/cm^2 in T-25 flasks, 35 mm, or 6-well culture plates.

MC3T3-E1 cells, a murine pre-osteoblast line capable of differentiation and mineralization in culture [20], were a gift from Dr. Norman Karin, University of Delaware. MC3T3-E1 cells were maintained in an MC3T3-E1 growth medium of alpha Minimum Essential Medium (αMEM) (Mediatech, Herdon, VA), 10% neonatal FBS (Cansera, Roxdale, Ontario), and penicillin 100 U/ml/streptomycin 100 μg/ml. For experiments as indicated, MC3T3-E1 cells were plated at 10^4 cells/cm^2 in MC3T3-E1 differentiation medium (αMEM, 10% FBS, penicillin 100 U/ml/streptomycin 100 μg/ml, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (Sigma)).

Breast cancer cells

MDA-MB-231 cells, a human metastatic breast cancer line derived from a pleural effusion [21], were a gift from Dr. Danny Welch, University of Alabama, Birmingham. The cells were maintained in a breast cancer growth medium of DMEM, 5% FBS, and penicillin 100 U/ml/streptomycin 100 μg/ml.

Primary osteoblasts from neonatal mouse calvariae

Osteoblasts were isolated from neonatal mouse calvariae and cultured as described [22]. Animal use was approved by the IACUC of the Pennsylvania State University. Briefly, 2 to 5 calvariae were dissected from 2 day, C57bl/6, mouse pups. The calvariae were rinsed with PBS, cut into pieces, and incubated with 4ml of digestion solution [0.64 mg/ml Collagenase type IA (Sigma) and 0.05% trypsin in PBS] at 37° for 20 min with shaking immediately before incubation and again after 10 min. After the 20-min incubation, the solution containing the cells was collected and 700 μl of FBS was added. The calvariae pieces were washed with 2 ml of DMEM and the wash added to the digestion solution containing cells, and centrifuged (300×*g*, 4 min). The cell pellet was resuspended in calvariae growth medium (DMEM, 10% FBS, penicillin 100 U/ml/streptomycin 100 μg/ml, 100 μg/ml ascorbic acid) and plated in a 6-well plate, 2 ml cell suspension per well (pool 1). This procedure was repeated three more times (pools 2–4). The following day, culture media were replaced with fresh growth medium and cells were monitored until ~85% confluency was reached. The cells were trypsinized [0.25% trypsin/2.21 mM EDTA in Hanks' Balanced Salt Solution (CellGro, Mediatech, Herndon, VA)]. Pools 1 and 2 were combined and pools 3 and 4 were combined. Cells were plated at a cell density of 1 to 2×10^4 cells/cm^2 in calvariae growth medium. The next day the culture media was removed and calvariae differentiation medium (DMEM, 10% FBS, penicillin 100 U/ml/streptomycin 100 μg/ml, 100 μg/ml ascorbic acid, 40 ng/ml dexamethasone) was added to the cells and changed twice a week until the desired osteoblast age was reached. Combined pools 3 and 4, 14 days from addition of differentiation medium, were used in experiments described in this study.

Conditioned media preparation

MDA-MB-231 breast cancer cells were grown to 90% confluency. Breast cancer growth medium was removed and the cultures rinsed once with PBS. DMEM:Ham's F12 (for use with hFOB 1.19 cells) or αMEM (for use with MC3T3-E1 cells) was added to the cancer cells (20 ml in a T-150 flask, ~1.3×10^5 cells/cm^2). Cultures were incubated for 24 h. Breast cancer cell conditioned medium (BCCM) was collected, centrifuged (300×*g*, 10 min) to remove cellular debris, and stored at −20 °C. hFOB 1.19 cell conditioned medium was prepared similarly. hFOB 1.19 cells (80% confluent) were rinsed one time with PBS before serum-free DMEM:Ham's F-12 1:1 and penicillin 100 U/ml/streptomycin 100 μg/ml were added, collected after 24 h of incubation, centrifuged to remove debris, and stored at −20 °C.

Conditioned media treatments of osteoblasts

Vehicle media (VM) consisted of differentiation media appropriate for each of the cell lines used. A 2× differentiation medium was formulated for each of the osteoblast cell lines. For hFOB 1.19, medium consisted of DMEM: Ham's F12, 20% FBS, 2×10^{-8} M 1,25 dihydroxyvitamin D_3, 2×10^{-8} M vitamin K, 100 μg/mL ascorbic acid, 200 IU/ml penicillin, and 200 μg/ml streptomycin. For MC3T3-E1, the medium consisted of αMEM, 20% neonatal FBS, 100 μg/ml ascorbic acid, 20 mM β-glycerophosphate, 200 IU/ml penicillin, and 200 μg/ml streptomycin. Conditioned media (CM) was comprised of one half volume BCCM and one half volume 2× osteoblast differentiation medium appropriate for the osteoblast line used in the experiment. This scheme ensured that concentrations of serum and differentiation factors were identical for VM and CM.

Peptides and cytokines

TGF-β and anti-TGF-β neutralizing antibody were purchased from R&D Systems, (Minneapolis, MN) and used at concentrations indicated. The antibody was incubated with VM or CM for 1 h at 37 °C prior to culturing with the osteoblasts. Parathyroid hormone (PTH) (1–34) was obtained from Sigma, St. Louis, MO.

Cytokine analyses

Cytokines in the culture medium were detected using RayBio® Mouse Cytokine Antibody Array III System for MC3T3-E1 and RayBio® Human Array System I for hFOB 1.19 cells (Norcross, GA). Cytokine protein levels were quantitated using sandwich ELISAs following the protocols recommended by R&D Systems. Intra-assay variation was typically less than 15%. Select cytokines were quantitated with Bio-Plex™ Mouse and Human Cytokine Assay System (Bio-Rad, Hercules, California).

Statistical analyses

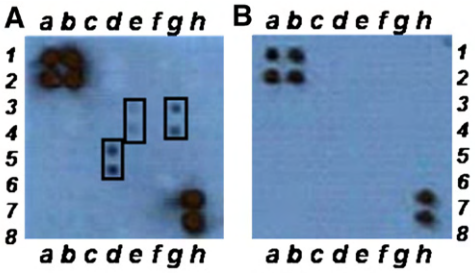
Statistical analyses were carried out using SAS, For Windows (SAS Version 9.1, SAS Institute, Cary, NC). Main effects were evaluated using one-way analysis of variance (ANOVA). Data were used for analyses of all variables. Statistical significance was defined as a probability $P<0.05$ in all analyses. *N* values for individual experiments are provided in the figure legends.

Results

The production of IL-6, IL-8, and MCP-1 by hFOB 1.19 cells was increased in the presence of breast cancer conditioned medium and was dose-dependent

When hFOB 1.19 were approximately 90% confluent, they were rinsed with PBS and treated with either VM or CM. Twenty-four hours later, the culture media were collected and screened with a RayBio® Human Cytokine Array. Among the 62 cytokines in the screen, IL-6, IL-8, and MCP-1 levels were readily detected (Fig. 1A). In this array, no cytokines were detected in the BCCM alone (Fig. 1B). Medium from VM-treated osteoblasts also had no detectable levels of cytokines in this array (data not shown).

In order to quantitate detected cytokine levels, standard ELISAs were carried out. The BCCM was assayed for the presence of human IL-6, IL-8, and MCP-1. In this assay, 73 pg/ml IL-6, 449 pg/ml IL-8, and <2 pg/ml MCP-1 were detected. These basal levels of cytokines were subtracted in the data that follow. At a later time, we assayed BCCM with a multiplex assay (Bio-Rad Bio-Plex™) and found 50 pg/ml IL-6, 51 pg/ml IL-8 and 1.6 pg/ml MCP-1.



RayBio® Human Array System I

	a	b	c	d	e	f	g	h
1,2	Pos	Pos	Neg	Neg	G-CSF	GM-CSF	GRO	GRO-α
3,4	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5,6	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
7,8	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

Fig. 1 – hFOB 1.19 secreted cytokines detected by a cytokine array. hFOB 1.19 cells were grown in growth medium at 34 °C until they reached approximately 80–90% confluency. Growth medium was removed, cells rinsed with PBS, and subsequently treated with VM or CM for 24 h at 34 °C. The resultant culture supernatant was collected, centrifuged to remove debris, and analyzed with a RayBio® Human Cytokine Protein Array System I. (A) Array results of culture supernatants of hFOB cells treated with CM. From left to right: positive control (a,b,1,2), MCP-1 (d5,6), IL-6 (e3,4), IL-8 (g3,4), positive control (h7,8). (B) Array results of MDA-MB-231 BCCM alone. Positive controls (a,b,1,2 and h7,8). No cytokines were detected.

To define the osteoblast cytokine response to BCCM and determine whether the stage of osteoblast differentiation was important for this response, hFOB 1.19 were treated with CM for 24 h after various days of culture. hFOB 1.19 were grown to confluency at 34 °C and on day 6 transferred to 39 °C to allow for differentiation. At days 3, 5, and 7 after transfer, the cells were treated with either VM or CM. Twenty-four hours later, the culture medium was collected and assayed by ELISA for human IL-6, IL-8, and MCP-1. On days 4 and 6, the hFOB 1.19 cells treated with VM contained undetectable levels of IL-6, while the osteoblasts treated with CM contained 0.11 ng/ml and 0.26 ng/ml IL-6 respectively (Fig. 2A). However, the differences were more pronounced as the osteoblasts differentiated. For example, a 24-h exposure of 8 day-differentiated osteoblasts to CM led to a 5- to 10-fold increase in IL-6 (~10 ng/ml) (Fig. 2A). At this time the cytokine levels of IL-6 from cells treated with VM increased to about 1 ng/ml.

A similar pattern was found with IL-8 (Fig. 2B). Cytokine concentrations in osteoblasts treated with VM were low (0.02 ng/ml) during the early differentiation stage; however, the concentration of IL-8 increased to 0.26 ng/ml by day 8. Treatment with CM increased osteoblast production of IL-8 by nearly five times (1.26 ng/ml) when compared to treatment with VM at the same time (0.26 ng/ml).

Osteoblast production of MCP-1 displayed a different pattern of expression than IL-8 and IL-6. MCP-1 increased in cells treated both with VM and CM. However, the concentrations of MCP-1 were higher in cultures of osteoblasts treated with CM than with VM (Fig. 2C). This increase in MCP-1 was

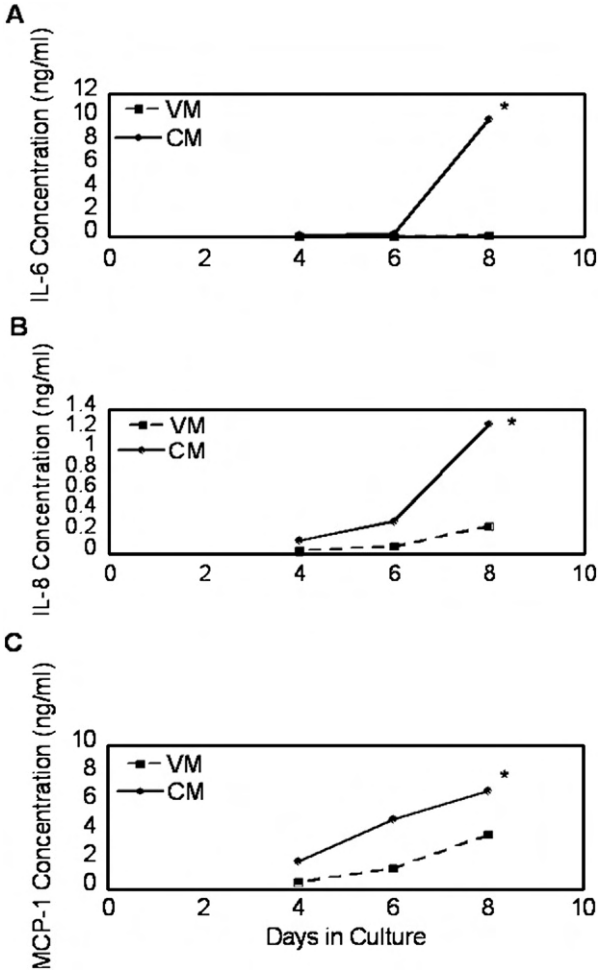


Fig. 2 – Increase in secretion of IL-6 by hFOB 1.19 at various stages of differentiation in the presence of breast cancer conditioned medium. hFOB 1.19 were grown at 34 °C until confluency (day 6) when they were changed to differentiation conditions as indicated in the methods section. Twenty-four hours prior to the indicated day, osteoblasts were treated with either VM or CM. The resultant culture supernatants were collected and assayed by ELISA. (A) IL-6; (B) IL-8; (C) MCP-1. The concentration of IL-6 in the BCCM alone was approximately 73 pg/ml, IL-8 was 449 pg/ml and MCP-1 was undetectable (<2 pg/ml). These baseline cytokine levels were subtracted from values obtained after hFOB treatment with CM. Experiment was performed twice for days 4 and 6 and eight times for day 8 with similar results. Shown is a representative experiment. By paired t-test, *P=0.004 for IL-6, and 0.001 for IL-8 in a total of 5 separate experiments. *P=0.022 for MCP-1 in a total of 4 separate experiments (P value calculations included data not shown).

maintained throughout all stages of osteoblast differentiation (day 4 osteoblasts treated with VM=0.47 ng/ml MCP-1, CM=1.92 ng/ml MCP-1; day 8 osteoblasts treated with VM=3.77 ng/ml MCP-1, CM=6.84 ng/ml MCP-1).

The dose response of CM-treated osteoblast cytokine expression was determined by treating hFOB 1.19 with 50%, 25%, and 12.5% BCCM. After 24 h, the resultant culture supernatants were collected and assayed by ELISA for IL-6, IL-8, and MCP-1. Osteoblast production of IL-6, IL-8, and MCP-1 increased as the percentage of BCCM was increased (Fig. 3).

To determine if the altered cytokine production was specific to breast cancer conditioned medium, osteoblast cultures were treated with osteoblast CM. There were no significant changes in osteoblast production of IL-6, IL-8, or MCP-1 compared to treatment with VM (data not shown).

Direct co-culture of hFOB 1.19 and MDA-MB-231 breast cancer cells

A direct co-culture model was tested to determine if the changes similar to treatment with CM occurred with cell contact. MDA-MB-231 cells were added to a differentiating (day 8 at 39 °C) monolayer of hFOB 1.19 at a ratio of 1:10, MDA-MB-231 to hFOB cells. MDA-MB-231 and hFOB 1.19 cells were cultured separately as controls. After 24 h, the culture supernatants were collected and assayed for IL-6 and IL-8 by ELISA. The supernatant from the osteoblast-cancer cell co-culture contained nearly 40 ng/ml of IL-6 and 10 ng/ml of IL-8, while the hFOB 1.19 cells alone expressed ~6 ng/ml IL-6 and ~3.5 ng/ml IL-8 (Fig. 4). Breast cancer cells cultured alone expressed

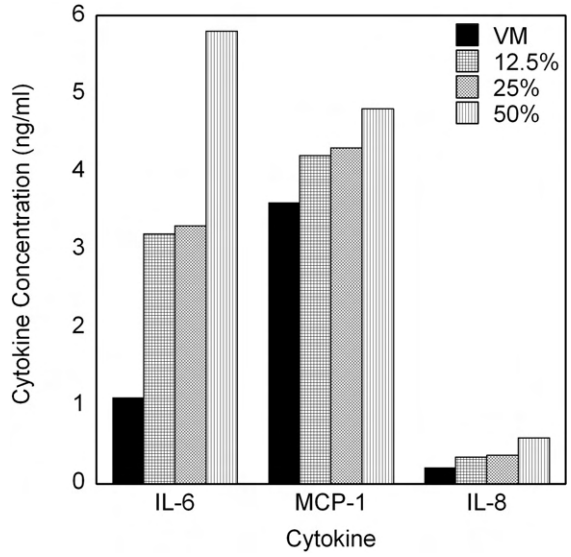


Fig. 3 – The hFOB 1.19 increase in cytokine secretion by breast cancer conditioned medium was dose dependent. hFOB 1.19 cells were plated and grown at 34 °C until confluency when they were changed to differentiation conditions as indicated in the methods section. On day 7, osteoblasts were treated for 24 h with VM or CM at either 50%, 25%, or 12.5% BCCM. The resultant culture supernatants were collected and cytokine levels determined by ELISA.

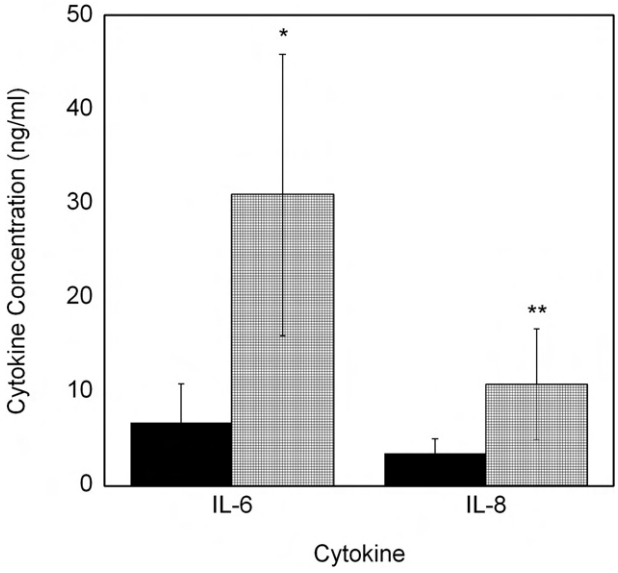
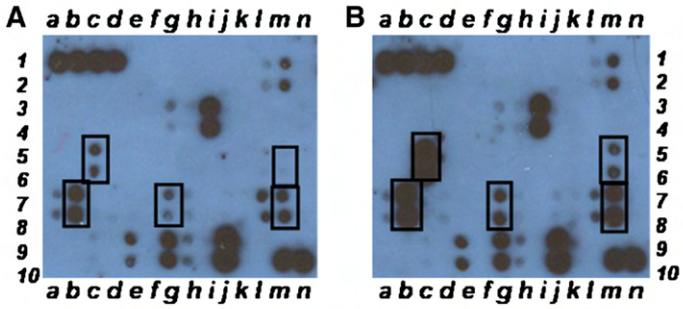


Fig. 4 – Increase in cytokine production by hFOB 1.19 co-cultured directly with MDA-MB-231 Cells. hFOB 1.19 were plated and grown at 34 °C until day 6 when the confluent cultures were moved to 39 °C. On day 7, cells were counted, and MDA-MB-231 metastatic breast cancer cells were added to the existing hFOB osteoblast cultures at a ratio of 1:10 (breast cancer cells: osteoblasts). MDA-MB-231 cells were also plated alone. Cultures were then incubated at 37 °C for 24 h. The resultant culture supernatants were collected and assayed by ELISA for IL-6 and IL-8. Medium from MDA-MB-231 cells cultured alone had undetectable levels of IL-6 and IL-8. Solid bars, hFOB 1.19 cells; checked bars, MDA-MB-231 cells co-cultured with hFOB cells at a 1:10 ratio. $N \geq 2$, $^{*}P < 0.005$; $^{**}P < 0.05$.

<200 pg/ml of IL-6 or IL-8. Therefore, there was a 6-fold increase in IL-6 and a 3-fold increase in IL-8 production under co-culture conditions. While we believe that the cytokines were produced by the osteoblasts, it is possible, in a co-culture situation, the osteoblasts may have stimulated the breast cancer cells to increase cytokine production.

MC3T3-E1 osteoblasts increased cytokine production in the presence of MDA-MB-231 conditioned medium

Due to the temperature-sensitive nature of the hFOB 1.19 cells and the complications of using two human cells lines, we also tested a murine osteoblast line, MC3T3-E1. In initial experiments, the cells were cultured for 25 days in MC3T3-E1 osteoblast differentiation medium before being treated with VM or CM for 24 h. The resultant culture supernatants were collected and assayed with a RayBio® Mouse Cytokine Array III system. There were visible increases in IL-6 and MCP-1 as well as LIX (CXCL 5), macrophage inflammatory protein-1 γ (MIP-1γ), and RANTES (Fig. 5). Similar increases were observed when the MC3T3-E1 cells were treated with CM after 12 days of culture, a time when the osteoblasts were in the early matrix-forming stage (data not shown). These assays were followed by murine ELISAs to confirm and quantify



RayBio® Mouse Array System III

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	POS	POS	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	NEG	NEG	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3,4	Eotaxin-2	Fas Ligand	Fracta-lkine	G-CSF	GM-CSF	IFN-γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	IL-2	IL-3	IL-3 Rb
5,6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L-Selectin
7,8	Lympho-tactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3β	MIP-3α	PF-4	P-Selectin	RANTES	SCF
9	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	Blank
10	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	POS	POS

Fig. 5 – Conditioned medium from breast cancer cells induced osteoblast cytokine production by MC3T3-E1 cells. After 12 or 25 days in culture, MC3T3-E1 osteoblasts were treated with either VM or CM. Twenty-four hours later, the resultant culture supernatants were collected and a RayBio® Mouse Cytokine Antibody Array III was used to detect changes in osteoblast cytokine production. Shown are the results from 25 days; 12-day results were similar. (A) Murine osteoblast VM cytokine production. (B) Murine osteoblast cytokine production after 24 h treatment with CM. From left to right: MCP-1 (b7,8), IL-6 (c5,6), MIP-1γ (g7,8), LIX (m5,6), and RANTES (m7,8). Osteoblast secretion of cytokines was both induced (LIX) and increased (MCP-1, IL-6, MIP-1γ, and RANTES) when treated for 24 h with CM.

MCP-1 and IL-6 expression. The increases in murine IL-6 were substantial (Fig. 6). For example, osteoblasts treated with VM for 6 h contained 0.72 ng/ml IL-6, whereas treatment for 6 h with CM yielded 4.32 ng/ml. The increases in murine MCP-1, however, were less dramatic (~4 ng/ml compared with ~7 ng/ml, data not shown). Since mice do not express IL-8, we assayed for the murine cytokine MIP-2, thought to function similarly to IL-8 [13]. MIP-2 was detected at low concentrations (~8 pg/ml) but increased with exposure to CM (~100 pg/ml, data not shown). Thus, exposure to BCCM elicited a similar cytokine response in both human and murine osteoblast lines. Additionally, an increased IL-6 response was demonstrated in primary mouse osteoblasts isolated from neonatal calvaria (Fig. 7). Due to the consistent dramatic increase in murine IL-6 production by the treated osteoblasts, IL-6 was designated as the signature cytokine for the inflammatory response.

To determine the response time to BCCM, MC3T3-E1 cells were cultured for 12 days and treated with VM or CM for 2, 4, or 6 h. The resultant culture supernatants were assayed for murine IL-6. As little as 2 h of exposure to the CM elicited a 2-fold increase in murine IL-6 secretion by the osteoblasts (VM treatment, 0.84 ng/ml IL-6; CM treatment, 1.8 ng/ml IL-6). After 6 h, the increase was nearly 5-fold (VM treatment, 0.72 ng/ml IL-6; CM treatment, 4.32 ng/ml IL-6) (Fig. 6).

Parathyroid hormone-related peptide, transforming growth factor-beta, and IL-1beta are candidate factors for mediating the expression of osteoblast cytokines

PTHrP, interleukin-1beta (IL-1β), and TGF-β are reported to be present in breast cancer conditioned media and have been reported to induce at least one cytokine of interest (IL-6, IL-8, or MCP-1) [23,24]. Therefore, they were considered as possible factors secreted by MDA-MB-231 cells that may promote osteoblast cytokine expression.

IL-1 β was not detected therefore, it was eliminated from further consideration. PTHrP was present at 46 pg/ml, approximately 2×10^{-12}M (Andrea Manni, Penn State Hershey). A similar value was reported by Guise et al. [24]. This concentration was too low to bring about an increase in IL-6 expression by the osteoblasts (data not shown). However, TGF-β was found to be present at between 1 and 5 ng/ml depending on the batch of conditioned medium (also see Mercer et al. [17]). These concentrations are sufficient to stimulate IL-6 production by the osteoblasts (see Fig. 8). We tested the

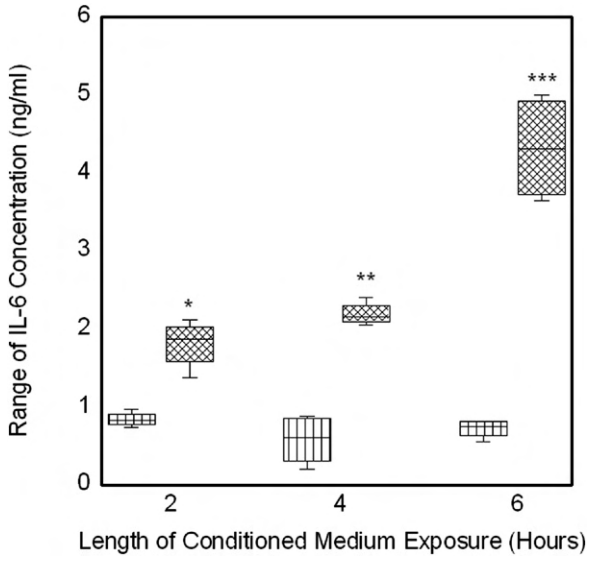


Fig. 6 – Conditioned medium from breast cancer cells rapidly induced osteoblast IL-6 production. After 12 days in culture, MC3T3-E1 osteoblasts were treated with VM or CM for 2, 4, or 6 h. The resultant culture supernatant was collected and murine IL-6 levels measured by ELISA. Shown is the range of IL-6 concentrations vs. hours of exposure to CM treatment in a box plot. Black bar, vehicle medium; checked bar, conditioned medium. N = 2 per condition. *P < 0.005 (2 h); **P < 0.0005 (4 h); ***P < 0.001 (6 h).

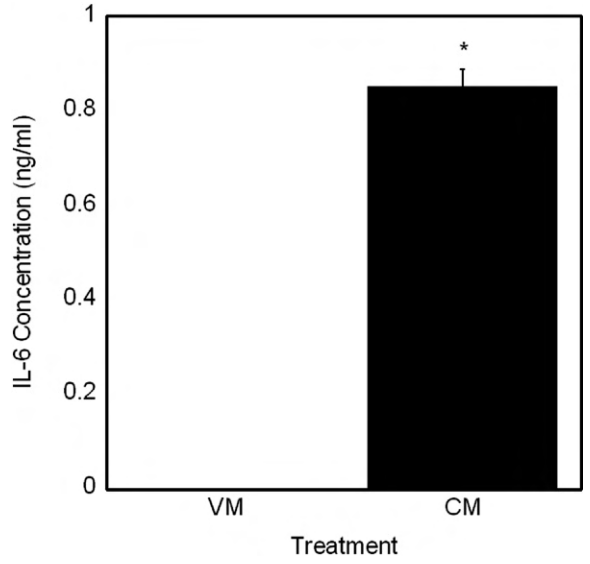


Fig. 7 – Primary murine calvarial osteoblasts secreted IL-6 in response to breast cancer conditioned medium. Primary osteoblasts were isolated from the calvariae of neonatal C57bl/6 mice as described in the methods section. The cells were cultured in calvariae differentiation medium for 14 days before the medium was changed to 50% VM or CM. After 24 h, culture media were collected and murine IL-6 was quantitated by ELISA. Shown are the means ± standard deviation. N = 3 per condition; *P < 0.001.

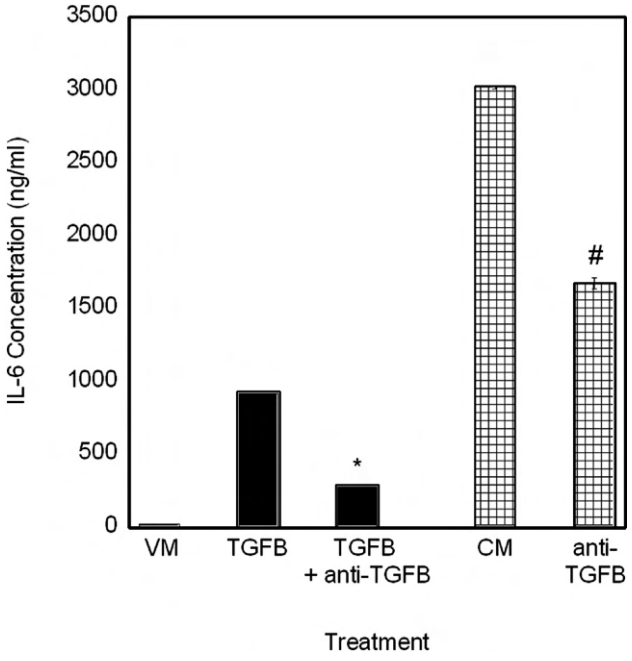


Fig. 8 – TGF-β is partially responsible for the expression of IL-6 by MC3T3-E1. MC3T3-E1 cells were cultured for 15 days. Conditioned medium from MDA-MB-231 cells, containing 1.4 ng/ml TGFβ, later determined by ELISA, was added to the cells at 50% or incubated for 1 h at 37° with 10 μg/ml neutralizing antibody to TGF-$\beta_{1,2,3}$ before addition. Other cultures were incubated with 2.5 ng/ml TGF-β or TGF-β that had been pre-incubated with 10 μg/ml antibody to TGF-$\beta_{1,2,3}$. One set of cultures was incubated with vehicle medium only. After 4 h incubation, the culture media were collected and assayed for IL-6. Black bar, vehicle medium; checked bar, conditioned medium. Shown are the means ± standard deviation of a representative experiment. This experiment was conducted three times in duplicate with similar results (N = 6 per condition); *P < 0.0003; #P < 0.0004.

combination of a suboptimal concentration of 0.5 ng/ml TGF-β with 5×10^{-12} M PTHrP and did not find a synergistic production of IL-6 (data not shown). Therefore, we focused on TGF-β in the breast cancer cell conditioned medium.

We determined the effect of adding a TGF-β neutralizing antibody to the breast cancer cell conditioned medium. Addition of conditioned medium to MC3T3-E1 increased their production of IL-6 nearly 200-fold compared to addition of regular differentiation medium (VM) (Fig. 8). Treatment of the conditioned medium with an excess (10 μg/ml) of neutralizing antibody to TGF-$\beta_{1,2,3}$ reduced osteoblast IL-6 expression by 45% compared with untreated conditioned medium. Addition of TGFβ (2.5 ng/ml) to the osteoblasts increased IL-6 production by approximately 50-fold. This value was reduced by 70% following incubation of the TGFβ antibody with the TGFβ before addition to the cells. We later determined that the conditioned medium contained approximately 1.4 ng/ml TGFβ. Thus, the antibody was far in excess of that required to neutralize 90% of the antigen. A similar result was seen when we used an inhibitor (Tocris, Ly364947) of the TGFβ receptor (data not shown).

Discussion

We found that osteoblasts responded to conditioned medium from MDA-MB-231 human metastatic breast cancer cells with enhanced cytokine expression. Specifically, the osteoblasts increased production of IL-6, MCP-1, and IL-8 (human), three cytokines characterized as osteoblast inflammatory stress proteins. These molecules may help to provide a favorable tumor cell environment as well as initiate osteoclastogenesis. This finding supports the idea that breast cancer metastases create a unique niche in the bone microenvironment by co-opting the normal cells of the bone to favor tumor growth and development. While other cells types are undoubtedly involved in the metastatic and tumorigenic process, here we demonstrate a direct effect of metastatic breast cancer cells on osteoblasts.

Breast cancer metastasis to bone is predominantly an osteolytic disease whereby osteoclasts, not cancer cells, are the cause of bone degradation. According to the "vicious cycle" paradigm [5], cancer cell-secreted PTHrP activates osteoblasts to express RANKL which binds to receptor activator of nuclear factor kappa B (RANK) on osteoclasts leading to their activation and subsequent bone loss. Osteoclast resorption of the bone matrix releases TGF-β that acts on the tumors cells to stimulate more PTHrP. However, osteoclasts are not solely responsible for sustained bone loss. Administration of bisphosphonates to inhibit osteoclast activity does not result in resolution of bone lesions [15]. Lesion formation is slowed but lesions do not heal. Clearly the impact of metastatic breast cancer cells on osteoblasts cannot be ignored.

In a closer examination of the fate of the osteoblasts, it was found that in the presence of MDA-MB-231 breast cancer cells or their conditioned media, cultured osteoblasts exhibited an increase in apoptosis, a change in morphology, and suppression in differentiation and mineralization as evidenced by a lack of expression of alkaline phosphatase, bone sialoprotein, and osteocalcin [16,18]. This increase in osteoblast apoptosis and decrease in alkaline phosphatase expression was also detected *in vivo* in a mouse model [25]. In addition to these phenotypic effects, the current study demonstrates that osteoblasts exhibit an increase in inflammatory cytokines in the presence of metastatic breast cancer cells. A cytokine array revealed that three cytokines in particular, IL-6, IL-8, and MCP-1, increased dramatically when hFOB 1.19 human osteoblasts were treated with CM. IL-6 and IL-8 were also elevated when hFOB 1.19 cells were co-cultured. MC3T3-E1 murine osteoblasts displayed a similar increase in murine IL-6 and MCP-1 when exposed to metastatic breast cancer cell conditioned media. As further evidence, we have also seen an increase in MIP-2 and KC (murine homologues to human IL-8 [13]) both *in vitro* and *ex vivo* (unpublished data). Exposure of primary murine osteoblasts to MDA-MB-231 breast cancer conditioned medium also elicited an increase in murine IL-6. These same cytokines are expressed in high levels by osteoblasts in debris-mediated osteolysis [26], which occurs when particles from prosthetic devices cause a chronic state of inflammation in the bone microenvironment [12]. Additionally, osteoblasts exposed to *M. tuberculosis* or *S. aureus* both *in vitro* and *in vivo* undergo an inflammatory stress response and produce IL-6, IL-8, and MCP-1 [10,27,28]. The infection of bone by these two microbes elicits the chronic inflammatory response and bone damage observed in trauma-induced osteomyelitis. Thus, the present study indicates that breast cancer cells evoke a similar stress response from osteoblasts.

IL-6 is a pleiotropic cytokine that influences many biological events including bone remodeling. In particular, IL-6 plays a role in the formation and activation of osteoclasts both *in vitro* and *in vivo* [29,30]. IL-6 has been implicated in the pathogenesis of bone resorption associated with Paget's disease [31], Gorham-Stout (disappearing bone disease) syndrome [32], and multiple myeloma [33]. Of interest to this study, IL-6 has been shown to induce production of PTHrP in human osteoblastic cells [34]. Soluble PTHrP, which is also derived from tumor cells [35], can stimulate the production of additional osteoblast-derived IL-6 through a feedback loop and facilitate osteoclastogenesis by decreasing the production of OPG and increasing osteoblast expression of RANKL. The bone resorption that subsequently follows releases stored TGF-β from the bone matrix that can in turn enhance breast cancer cell production of PTHrP [35].

In addition to playing a role in osteoclastogenesis and bone resorption, IL-6 has other functions that may contribute to cancer progression. Although the involvement of IL-6 in regulating the growth and apoptosis of breast cancer cells is unclear, IL-6 is known to be a growth factor for myeloma cells [36] and acts as an anti-apoptotic factor for human esophageal carcinoma and multiple myeloma cells [37,38]. In addition, IL-6 downregulates the expression of CXCL10, a chemokine with anti-malignant properties, by MDA-MB-231 cells [39]. IL-6 has also been reported to enhance the migration of T47D breast cancer cells *in vitro* [40]. Furthermore, the importance of IL-6 in cancer progression was demonstrated by a study in which IL-6 signaling in MDA-MB-231 breast cancer cells was blocked. This inhibition resulted in significantly decreased tumor engraftment, size, and metastasis in a nude mouse model [41]. Finally, IL-6 levels in breast cancer patients have been correlated with clinical stage [42,43] and rate of recurrence [44]. In particular, high IL-6 serum levels in patients with advanced or recurrent breast cancer were found to be an unfavorable prognosis indicator [45–47].

Besides acting on cells in the tumor/bone microenvironment, IL-6 has also been found to increase osteoblast production of another cytokine identified in our study: MCP-1. A monomeric polypeptide member of the CC chemokine superfamily [48], MCP-1 is a principle cytokine involved in inflammation and bone remodeling. MCP-1 is known to recruit cells involved in inflammation [13], osteoclast precursors [13], and angiogenesis [49]. These three processes are clearly involved in breast cancer tumorigenesis and lesion formation in the bone. MCP-1 is also normally produced by osteoblasts [50], but also is increased in metastatic cell lines [51].

MCP-1 has been found to be particularly important in cancer cell migration and metastasis. In a study that utilized the metastatic PC3, LNCaP, and bone metastatic LNCaP C4-2B prostate cancer cells, it was found that MCP-1 increased proliferation and invasion [52]. Interestingly, the G-protein-coupled receptor CCR2, the receptor for MCP-1, was found to be present on all the prostate cancer cell lines examined [52]. In addition, prostate cancer cells were found to produce high

levels of MCP-1 compared to primary prostate epithelial cells [53]. In that same study, MCP-1 was shown to mediate prostate cancer tumor-induced osteoclastogenesis and bone resorption [53]. In breast and ovarian cancer patients, MCP-1 serum levels have been correlated with advanced tumor stage [54,55]. In addition, the expression of MCP-1 in squamous cell carcinoma of the esophagus was equated with venous invasion and metastasis [56]. Furthermore, it has been shown that MCP-1 acts as a chemoattractant for myeloma cells [57]. Thus, it is evident that MCP-1 is a key mediator involved in inflammation and cancer cell progression.

IL-8 is a CXC inflammatory cytokine produced by many cell types including osteoblasts [50,58]. IL-8 was first identified as a neutrophil chemoattractant and is now known to attract monocytes and osteoclast precursors as well as promote angiogenesis [13,59]. In the bone, IL-8 has been shown to directly inhibit alkaline phosphatase expression [60] as well as decrease normal bone resorption and increase the motility of osteoclasts to new resorption sites [61]. Interestingly, human osteoclasts have also been shown to secrete high levels of IL-8, indicating the molecule's importance in normal bone remodeling [62].

Along with IL-6 and MCP-1, IL-8 has also been found to be important in cancer cell progression. IL-8 is secreted by many tumor cell lines that are metastatic and osteolytic [63]. In addition, IL-8 appears to play a role in cell motility, invasion, and metastatic potential in human tumors [64–66]. Concerning this present study, increased bone metastatic potential of human breast cancer cells has been associated with the cancer cell's ability to express IL-8. In several studies done by Bendre et al. [58,63,67], metastatic breast cancer cell-derived IL-8 was found to directly stimulate osteoclastogenesis via RANKL dependent and independent mechanisms. In particular, it was proposed that IL-8 plays an important role in the "vicious cycle" of breast cancer cell metastasis to the bone. Breast cancer cell-derived IL-8 increased RANKL expression on osteoblasts, which in turn, facilitated osteoclast formation. In addition, soluble IL-8 directly promoted osteoclastogenesis, leading to bone resorption. Stored TGF-β is then released from the bone matrix that continues to drive the "vicious cycle," amplifying osteolysis and supporting breast cancer bone metastases [58]. IL-8 has also been implicated in the clinical outcome of patients with breast cancer. In a study involving 69 women with operable or advanced breast cancer, elevated serum IL-8 levels were found to be directly associated with the clinical stage of breast cancer and were found to be indicative of a poor prognosis [42,68].

Do these cytokines feedback directly onto the tumor cells? It seems unlikely since the MDA-MB-231 cells do not have receptors for these cytokines. Specifically, as reported in the literature, MDA-MB-231 cells do not express the CXCR1 receptor for IL-8, [63] but do express low levels of CXCR2 mRNA [69]. Nonetheless, neutralizing antibody to IL-8 did not affect the growth of the MDA-MB-231 cells [70]. MDA-MB-231 cells do not express receptors for IL-6 [71,72]. This finding is consistent with their lack of response to IL-6 [73]. There is one report of low expression for the mRNA for the receptor for MCP-1, but none for the protein itself [74]. These reports taken together indicate that the cancer cells would not be expected to respond to these cytokines.

Our hypothesis is that osteoblasts produce these cytokines in response to the cancer cells, and that these cytokines then go on to activate osteoclasts. It is well documented in the literature that IL-6, IL-8, and MCP-1 have osteoclast stimulating properties [67,75–77].

In summary, we have shown that osteoblasts display an inflammatory response when exposed to breast cancer cell conditioned medium. The inflammatory cytokines, IL-6, MCP-1, and IL-8 can target osteoclast precursors and osteoclasts to bring about bone matrix destruction. Thus, osteoblasts contribute to the osteolytic phenotype, both through suppression of bone deposition and production of cytokines that recruit and activate osteoclasts.

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The bone microenvironment in metastasis; what is special about bone?

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Abstract The skeleton is a common destination for many cancer metastases including breast and prostate cancer. There are many characteristics of bone that make it an ideal environment for cancer cell migration and colonization. Metaphyseal bone, found at the ends of long bone, in ribs, and in vertebrae, is comprised of trabecular bone interspersed with marrow and rich vasculature. The specialized microvasculature is adapted for the easy passage of cells in and out of the bone marrow. Moreover, the metasphyseal regions of bone are constantly undergoing remodeling, a process that releases growth factors from the matrix. Bone turnover also involves the production of numerous cytokines and chemokines that provide a means of communication between osteoblasts and osteoclasts, but co-incidentally can also attract and support metastatic cells. Once in the marrow, cancer cells can interact directly and indirectly with osteoblasts and osteclasts, as well as hematopoietic and stromal cells. Cancer cells secrete factors that affect the network of cells in the bone microenvironment as well as interact with other cytokines. Additionally, transient cells of the immune system may join the local mileau to ultimately support cancer cell growth. However, most metastasized cells that enter the bone marrow are transient; a few may remain in a dormant state for many years. Advances in understanding the bone cell-tumor cell interactions are key to controlling, if not preventing metastasis to bone.

Keywords Bone metastasis · Osteoblasts · Osteoclasts · Cytokines · Chemokines

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1 Tumor cell metastasis

The skeleton is a favored site of metastasis for a number of common tumors. Bone metastases are by far more prevalent than primary tumors of the bone. Based on post-mortem examination, approximately 70% of patients who die from breast or prostate cancer have bone metastases [1]. The incidences from thyroid, kidney, and lung cancer also are high (about 40%). In contrast, it has been noted that bone metastases from cancers of the gastrointestinal tract are uncommon. In many cases, cancer cell metastases are diagnosed in patients before diagnosis of the primary disease. A better understanding of the specificity and the pathogenesis of metastasis will allow for better therapeutic treatments and quality of life for patients.

The metastasis of a primary tumor to distant organs requires a series of coordinated steps. Proliferation of the primary tumor is supported by tumor autocrine factors or local growth factors, such as vascular endothelial growth factor (VEGF), tumor growth factor-beta (TGF-β), and interleukin-6 (IL-6). For a tumor to reach a clinically detectable size, localized neovascularization or angiogenesis must occur. The development of new blood vessels provide an endless supply of nutrients as well as a route for tumor cell migration to secondary sites. Subsequently, local

invasion takes place, which is accomplished by the destruction of the extracellular matrix, including the basement membrane and connective tissue. The process of invasion through a basement membrane is a hallmark characteristic of a metastatic cell. Additionally, tumor cells experience increased motility and reduced adherence allowing them to migrate into lymph or blood vessels. Intravasation into blood vessels at the primary site may occur as a result of excess force or response to a soluble chemotactic factor gradient. After circulating through the vasculature, tumor cells may adhere to vessel endothelium of the target organ and extravasate into the tissue. This movement is facilitated by cancer cell secretion of matrix metalloproteinases (MMPs) and cathepsin-K that destroy surrounding tissue. Finally, tumor cells thrive at the secondary site, a defining characteristic of metastatic tumor cells, only if there is an appropriate environment of paracrine or autocrine factors that aid in growth and vascularization [2–4]. The distribution pattern of cancer cells to the bone is believed to be due to the venous flow from breast and prostate towards the vena cava and into the vertebral venous plexus [5]. Once in the circulation, entry of the cancer cells into the venous circulation of the bone marrow may be facilitated by the slow blood flow and particular anatomy of the venous sinusoids. Nonetheless these steps alone do not explain survival and growth of the cancer cells in the bone.

2 Bone structure

In order to understand the bone-tumor microenvironment, one must consider bone structure and function. Bone is a specialized type of connective tissue, which provides structural support, protective functions, and plays a major role in the regulation of calcium levels in the body [6]. Type I collagen accounts for 95% of the organic bone matrix [7]. The remaining 5% includes proteoglycans and a variety of other non-collagenous proteins [6]. This largely collagenous matrix is hardened through the mineralization process, in which hydroxyapatite ($3Ca[PO_4]_2[OH]_2$) crystals are deposited in the organic matrix [8]. Mineralization increases bone resistance to compression [9], and also contains numerous growth factors, including TGF-β, which are released upon bone resorption [10].

The bones of the body are classified as long bones (e.g. the tibia, femur, and humerus) and flat bones (e.g. the skull, ileum, and mandible). Both types contain cortical and trabecular bone, albeit in different concentrations. Cortical bone, the compact, dense outer protective layer of bone, is made up of tightly packed collagen fibrils [6]. This form of bone is vital for supporting the weight load of the body. On the other hand, trabecular bone, also known as cancellous bone, has a loosely organized, porous matrix and is located in the interior of bone, near the ends. Trabecular bone is metabolically active. All bone matrix undergoes remodeling, but trabecular bone has a greater turnover rate than cortical bone [6].

Long bones are divided into the diaphysis, metaphysis, and epiphysis in a growing individual [11] (Fig. 1). The long bone ends, or epiphyses, are located above the growth plate, where bone elongation occurs. The diaphysis, which is the long, narrow shaft of the bone, is primarily composed of cortical bone. The metaphysis, located near the ends of bones just below the growth plate, is predominantly composed of trabecular bone and is surrounded by hematopoietic marrow, fatty marrow, and blood vessels [11].

3 Cells in the bone microenvironment

Bone is a dynamic structure that undergoes constant remodeling in order to respond to mechanical strain and maintain calcium homeostasis. Bone resorption and deposition occur in a tightly regulated fashion that is orchestrated by three cell types: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are derived from mesenchymal stem cells located in the bone marrow stroma. They synthesize osteoid (i.e. new bone matrix), comprised primarily of collagen and non-collagenous proteins, and also aid in mineralization of the bone matrix. Upon stimulation by bone morphogenetic proteins and local growth factors, the mesenchymal stem cells proliferate and form pre-osteoblasts, which subsequently differentiate into mature osteoblasts [12]. After synthesizing new bone

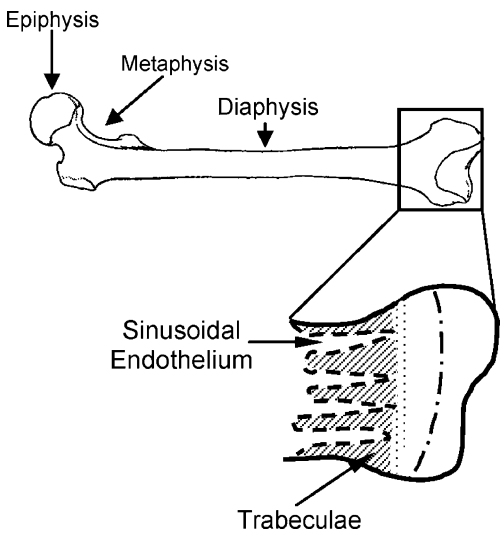

Fig. 1 Diagram of a long bone indicating the major regions and the major structures within the metaphysis, i.e. trabecular bone and the sinusoidal endothelium

matrix, the osteoblast either undergoes apoptosis or becomes embedded in the bone as an osteocyte [13]. These cells have long processes that allow them to remain in contact with other osteocytes and with osteoblasts that line the bone surface. The processes connect the entire matrix through a series of canaliculi [14, 15].

Osteoclasts, responsible for bone resorption, are derived from monocytes in the bone marrow [16]. Monocytes are activated to form osteoclasts through osteoblasts. Osteoblasts express the receptor-activator for NF-κB ligand (RANK-L) on their external surfaces; RANK-L binds to the receptor RANK found on the surface of monocytes. In the presence of macrophage colony stimulating factor (M-CSF), RANK-L promotes cellular fusion of several monocytes to form a multinucleated osteoclast [16]. Activated osteoclasts bind to the bone matrix through $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$ integrins located on the membrane surface and also secrete acid and lysosomal enzymes which degrade bone [13, 16].

Other cell types located within the bone microenvironment may also contribute to the bone metastatic niche. These cells can generally be grouped into two categories: stromal and transient. Mesenchymal stem cells in the bone marrow give rise to stromal cells which can differentiate into adipocytes, fibroblasts, chrondrocytes, or osteoblasts. Stromal cells have been found to support the differentiation, proliferation, and survival of both hematopoetic and cancer cells. In particular, it has been found that stromal cells express vascular cell adhesion molecule (VCAM-1). Michigami and colleagues discovered that the presence of VCAM-1 on stromal cells increased the production of bone-resorbing cytokines by myeloma cells. Neutralizing antibody to VCAM-1 or to $\alpha_4\beta_1$ integrin reduced osteolysis [17]. The adipocyte has been found to secrete tumor necrosis factor-alpha (TNF-α), IL-6, and leptin, which stimulate bone resorption and inhibit osteoblast proliferation [18, 19]. Factors secreted by adipocytes have also been implicated in breast cancer proliferation, invasiveness, survival, and angiogenesis [19]. Another type of mesenchymal cell, the fibroblast, has been shown to affect breast cancer cell invasion and contribute to metastatic bone disease. Fibroblast-secreted syndecan-1 was found to increase breast cancer cell proliferation *in vivo* [20]. In addition, fibroblasts secrete inactive MMP-2, which can be activated by breast cancer cells to subsequently increase their invasiveness and migration [21]. Fibroblasts have also been found to stimulate osteoclasts, and thus bone resorption, in a RANK-L dependent manner [22].

Vascular endothelial cells contribute to the formation of a favorable cancer cell microenvironment. New blood vessels, which arise from endothelial cells, are essential for the survival of cancer cells. Investigators have shown that the bone marrow, which has a high microvessel density, is associated with increased bone-tumor metastasis and survival of tumor cells [23]. It has also been found that many tumor-cell secreted factors stimulate endothelial cell proliferation, differentiation, and angiogenesis [23], thus producing a feedback loop that facilitates tumor cell survival in a secondary location.

Transient cells also contribute to the metastatic bone microenvironment. These cells include erythrocytes, T cells, and platelets, all derived from hematopoietic stem cells. In one study it was shown that platelets were directed by MDA-MB-231, a metastatic breast cancer cell line, to secrete LPA (Lysophosphatidic acid ([1-acyl-sn-glycero-3-phosphate]), a phospholipid with diverse biological activities [24]. Overexpression of this molecule and its receptor has been shown to increase tumor growth and metastasis [25]. Platelets may also adhere to cancer cells in the blood stream, allowing them to evade natural killer immune cell surveillance [26]. In addition, platelets may aid in cancer cell attachment to vascular endothelial cell walls [26]. T lymphocytes have been found to express RANKL, also known as TRANCE, and aid in osteoclast formation and activation [16, 27]. Peripheral T cells also secrete TNF-α, which is involved in osteoclastogenesis, inhibits osteoblast cell differentiation, and is a pro-apoptotic factor for osteoblasts [27, 28]. Besides enhancing osteoclastic bone resorption, T cells may be affected by bone metastatic cancer cells as well. As bone resorption is enhanced, TGF-β is released from the bone. This factor can inhibit both T cell proliferation and activity, and natural killer cell function [29]. Thus the immune response is suppressed and tumor cells may escape surveillance. In addition, tumor cell-secreted parathyroid hormone related peptide (PTHrP) and IL-8 may activate T cells, thus enhancing bone resorption and suppressing T cell function [29]. Plasma cells, or antibody-producing B cells, upregulate the receptor CXCR4 upon completion of differentiation [27]. Breast cancer cells, as well as stromal cells, express the ligand to this receptor CXCL12 [Stromal-derived factor-1 (SDF-1)] [27]. This ligand-receptor interaction may facilitate cancer cell migration into and within the bone microenvironment.

Tumor-associated macrophages (TAMs) are an important component of the inflammatory response in tissues [30]. These cells are derived from monocytes and are recruited by monocyte chemotactic peptide (MCP) chemokines [30]. Although activated TAMs kill many cancerous cells through secretion of IL-2, interferon, and IL-12, they also secrete a variety of potent angiogenic and lymphangiogenic growth factors, cytokines, and proteases [31, 32], all of which are involved in the promotion of tumor cell growth and survival. TAMs, as well as tumor cells, produce IL-10 which suppresses the anti-tumor response of cytotoxic T cells [33]. TAMs also induce the expression of VCAM-1 on mesothelial cells, which can aid in tumor cell invasion [34].

4 Bone remodeling

Mineralized bone has an abundance of growth factors, calcium ions, cell adhesion molecules, cytokines, and chemokines that, when released into the microenvironment during bone remodeling, make the skeleton an attractive site for metastatic cancer cells. This observation was first described in 1889 by Stephen Paget, who recognized the nonrandom movement of cancer cells within the body that was unexplained by blood flow. Paget stated "When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil" [35]. This 'seed and soil' hypothesis helps to explain the preferential metastasis of certain types of cancers to the bone microenvironment, which provides a fertile soil where cancer cells can grow. Osteoclasts further contribute to this environment by acting as plows to break up the 'soil' and release its 'nutrients' for cancer cell growth and maintenance. Studies have shown that there is a close relationship between bone resorption and tumor cell growth [36].

The relative activities of osteoblasts and osteoclasts are normally tightly coupled in order to maintain a balance between bone formation and degradation. Bone remodeling is regulated both by systemic hormones and locally produced cytokines [37]. Cells in the bone marrow, especially stromal and immune cells, produce cytokines and growth factors that influence the activities of osteoblasts and osteoclasts [38]. However, this balance between bone synthesis and resorption is disturbed in several pathological conditions, including osteoporosis, rheumatoid arthritis, and skeletal metastases, resulting in osteoclast activity in excess of bone deposition by osteoblasts with net bone loss [38].

Osteoclasts likely prime the bone microenvironment for tumor cell growth through bone resorption. Although there is no definitive evidence linking increased bone resorption to increased tumor cell mass, a variety of studies have been carried out with bisphosphonates to investigate this relationship. Bisphosphonates are inorganic pyrophosphates with powerful inhibitory effects on bone resorption. One of their main targets is the osteoclast. Powles et al. showed that the administration of a bisphosphonate, clodronate, was associated with both decreased bone metastasis and death rate in patients with breast cancer [39]. Risedronate was found to reduce tumor burden in addition to osteolytic bone lesions in a nude mouse model [40]. Furthermore, nude mice treated with neutralizing antibodies to PTHrP (key factor involved in the 'vicious cycle' of bone metastasis), also experienced a decrease in tumor burden compared to controls [41, 42]. Even though inhibitors to bone resorption seem to reduce tumor burden in bone, the same does not hold true for soft tissues [39]. In the study carried out by Powles et al., the idea that inhibitors of osteolysis slowed tumor growth in soft tissue was refuted [39]. In addition, there are pre-clinical data suggesting that bisphosphonates have no effect on tumor burden in soft tissues if the drugs are administered after the metastases are already formed [3]. Taken together, these studies illustrate the importance of osteoclasts and the uniqueness of the bone microenvironment for tumor cell growth.

In patients with bone metastatic cancer, usually one of two types of lesions predominate: osteolytic or osteoblastic, although mixed lesions may occur especially early on [36]. During osteolytic metastases, typical for breast cancer metastases, osteoclastogenesis and osteoclast activation results from direct and indirect actions by metastatic cancer cells. Increased bone resorption results. Mastro and colleagues additionally found that bone metastatic breast cancer cells suppress osteoblast function, which includes decreased matrix deposition, decreased proliferation, altered adhesion, and loss of osteoblast differentiation [43–45]. These phenomanae together favor a microenvironment of increased bone turnover with decreased bone deposition similar to that seen in osteoporosis.

On the other hand, cancers such as prostate cancer tend to be predominantly osteoblastic in nature. Excess bone deposition occurs but not necessarily in an ordered fashion. While little is known about the exact mechanisms, endothelin-1 has been implicated in osteoblastic breast cancer metastases [46, 47]. Endothelin-1 has been found to stimulate the formation of new bone through osteoblast proliferation [48], and serum endothelin-1 levels were found to be increased in patients with osteoblastic prostate cancer metastases [49]. In addition to endothelin-1, platelet derived growth factor-BB (PDGF-BB) may also be a mediator for osteoblastic metastases. In a study done by Yi et al., increased expression of PDGF-BB by human metastatic breast cancer cells was correlated with increased bone formation [50]. The exact mechanism has yet to be determined. Regardless of the lesion type, these observations underscore the importance of crosstalk between cancer cells and the bone microenvironment which facilitates bone metastases.

Clinically, patients with breast cancer and other osteolytic metastases but also with osteoblastic prostate bone metastases are treated with bisphosphonate drugs that block osteoclast activity. However, therapies utilizing bisphosphonates, such as ibandronate (Boniva™), are not curative [51]. Lesion progression is slowed, but the pre-existing lesions do not heal [52–55]. Severe bone pain, fractures, hypercalcemia, and spinal cord compression may still occur [3, 10, 56, 57]. The inability of bone to regenerate following bisphosphonate treatment supports the *in vitro* finding that breast cancer cells alter osteoblast function in addition to stimulating osteoclast activity.

Not all bones of the skeleton are equally favored for metastases. The spinal vertebrate, ribs, and the ends of long

bones are preferred destinations of metastases. In general, well vascularized areas and areas of the skeleton containing red marrow are the sites of metastatic colonization. In osteoblastic metastases, bone deposition is usually made on trabecular bone surfaces without prior removal of old bone, but it may also be at sites of prior resorption. In myeloma, a malignancy of B cells, the plasma cells accumulate in the bone marrow and lead to osteolysis. Activation of osteoclasts and bone resorption far exceed bone deposition in this disease. Whether the lesions are overall lytic or blastic, the outcome is bone pain, pathological fracture, nerve compression syndromes and hypercalcemia [58].

5 The metaphyseal region of the bone has unique properties that distinguish it from the diaphysis

Scintigrams of humans with advanced disease clearly indicate that highly vascularized metaphyseal bone is a preferred site for secondary metastasis [1]. A trafficking study with a nude mouse model of metastatic breast cancer revealed that within 2 h of an inoculation of a bolus of metastatic breast cancer cells into the left ventricle of the heart, cancer cells were detected throughout the femur. However, by 24 h they had been cleared from the shaft (diaphysis) but remained at the metaphysis where they grew into large tumor masses [59]. The unique properties of the bone metaphysis make it an attractive site for metastatic cancer cells.

6 Metaphyseal bone: structure and vascularity

Metaphyseal bone is a highly vascularized structure found near the ends of long bone, in ribs and in vertebrate. It is composed of a network of thin bone spicules, sometimes referred to as “spongy bone.” In long bones, these spicules appear as mineralized fingers interspersed with red marrow and are in close proximity to the blood supply. The marrow contains hematopoietic, mesenchymal, and stromal cells. The vascular supply is sinusoidal in nature rather than a bed of capillaries. Lining the trabecular bone surfaces are osteoblasts and bone lining cells which share many properties [60]. Bone lining cells are believed to differentiate into osteoblasts when necessary for bone remodeling. The osteoblasts, as well as the marrow cells, provide an environment rich in growth factors, cytokines and chemotactic factors. These factors, and the vascular structure of the trabecular bone, are crucial for metastatic cancer cell colonization and growth.

The interactions of metastatic breast cancer cells with the vasculature has recently been well documented by Glinsky [61] and is only briefly summarized here. Metastatic foci are often seen where the sinusoid microvasculature is abundant [62]. This phenomenon is likely related to the unique anatomic, hemodynamic, and epithelial properties of the metaphyseal vascular bed. For one, the vasculature does not end in capillaries of small diameter as in most tissues. Instead it consists of voluminous sinusoids with lumens many times the diameter of cancer cells [63]. The sinusoids are within a few microns of the trabecular bone [64]. This unique structure leads to a sluggish flow of blood compared to that seen in the capillary networks of most other tissues [63]. For example, Mazo et al. found the blood flow in venous sinusoids of mouse calvaria to be 30 fold lower than the arterial rate. In another animal model, blood flow rates in canine long bone were assessed with microspheres [65]. It was found that metaphyseal and marrow cavity flow rates in sinusoids were 7–14 ml/min/g tissue, much slower than more rapidly metastasizing tissue such as the post-prandial intestine [66]. Thus cells entering the sinusoids are more “in a lake than in a stream.” In addition, sinusoids are specialized to allow easy movement of hematopoietic cells in and out of the marrow. The walls of the sinusoids are trilaminar and their structure helps explain why tumor cells can easily enter and leave [63]. Stromal endothelial cells line the sinusoidal lumen. These cells do not have tight junctions but may overlap or interdigitate. They rest on a basement membrane, the middle layer, which is irregular and discontinuous. The third layer, facing the bone marrow, is composed of adventitial cells, a type of phagocytic cell, which also do not form a tight layer. Thus the nature of the sinusoidal walls allows for easy two-way movement of hematopoietic and lymphoid cells. This structure is used advantageously by cancer cells [67].

Nevertheless, cellular entry into the sinusoids and migration into the marrow are not sufficient to insure colonization by the cancer cells. In a mouse study, it was observed that many more cancer cells entered the marrow cavity of the femur than remained to colonize it [59]. Presumably, many metastatic cancer cells in the blood can circulate through the bone, but few remain. Cancer cells, similar to leukocytes, migrate through the vasculature using a process of attachment-detachment through cell-adhesion molecules. The endothelial cells of the bone sinusoids constitutively and simultaneously express an array of tethering and adhesive proteins including P-selectin, E-selectin, intercellular adhesion molecule (ICAM-1) and VCAM-1. The vasculature of other tissues only express these molecules when stimulated by inflammatory cytokines [63]. Moreover, vasculature in one part of the bone may be different than other parts. Indeed Makuch et al. [68] found expression of P-selectin, E-selectin, ICAM and VCAM by vascular endothelial cells isolated from trabecular bone and from diaphyseal bone. However the endothelial cells from the trabecular bone but not diaphy-

seal bone showed a significantly increased expression in E-selectin when exposed to conditioned medium from immature osteoblasts. These data can be interpreted to suggest that osteoblasts of immature, metabolically active bone enhance E-selectin expression by nearby endothelial cells. This increase in cell attachment molecules would in turn enhance cancer cell extravasation into the bone marrow. Furthermore as discussed further on, inflammatory cytokines produced by osteoblasts in the presence of breast cancer cells may cause an even greater increase in cancer cell migration. In complementary approaches, others found that prostate cancer cells showed increased adherence to bone marrow microvasculature endothelium than from endothelium of other anatomical sites [69, 70]. Similar findings were reported for breast cancer [71, 72].

7 Adhesion molecules of the vascular endothelium

There has been an ongoing discussion of the roles of adhesion vs. entrapment in the movement of cancer cells into organs. The "leaky" vasculature suggests that entrapment is not the limiting event in bone metastasis. To the contrary, adhesion of metastatic cancer cells to the endothelium appears to play a specific and critical role. Evidence for the role of adhesion molecules has been found, both with prostate and breast cancer cells, which may explain their predilection to the bone [61]. For tumor cells to reach the bone marrow there must be a selective adhesion of the circulatory tumor cells to the endothelium of the bone marrow sinusoids. Therefore the adhesion molecules of the endothelium are of utmost importance.

The movement of cancer cells across the endothelium in the bone marrow has been likened to the movement of leukocytes across the endothelium. While the general patterns are likely the same, the actual molecules involved may differ [73–75]. The reported roles of various adhesion molecules may relate to the particular system, i.e. primary or secondary tumors and the specific organ. For example, selectin-mediated binding of colon cancer cells has been demonstrated to be important for their adhesion to the hepatic microvasculature [76]. This association may not hold for other metastatic tumors [61]. Makuch et al. [68] reported that active osteoblasts influenced E-selectin (but not P or S-selectin) expression on metaphyseal endothelium. The expression of E-selectin depended both on the stage of differentiation of the osteoblasts and the source of the microvasculature endothelium within the bone marrow. Galactin-3 is another molecule that participates in tumor cell, bone microvasculature associations. Galactin-3 and its ligand Thomsen–Friedenreich (TF) antigen are found both on many cancer cells and on microvasculature endothelium. Their interaction appears to be important for the primary arrest of the tumor cells [71, 77]. Another well studied molecule is CD44, the principle cell surface receptor for hyaluronic acid (HA). It is frequently over-expressed on malignant cells. In model systems, its expression correlates with the rate and strength of cancer cell interaction with bone marrow endothelium. CD44 expression on the surface of bone marrow endothelial cells likely acts to bind HA. Cancer cells and bone marrow endothelial cells both appear to express CD44 and HA, and the interaction of the two leads to tethering of the cancer cells to the bone marrow. In addition, there are associated data to suggest that activation of CD44 by HA or by osteopontin is important in downstream signaling through CD44 in bone.

8 Adhesion molecules within the bone marrow cavity

Coordinated bone remodeling involves extensive cell–cell and cell–matrix interactions among osteoblasts, osteoclasts, and bone marrow resident stromal and hematopoietic cells. The sinusoidal endothelium of the bone marrow is a two-way gate, allowing movement in both directions of newly formed and recirculating lymphocytes, hematopoietic stem cells as well as neoplastic cells. The trafficking patterns are organized by adhesion molecules on the circulating cells as well as on the bone marrow reticulocytes. VCAM-1, a member of the immunoglobulin family of cell adhesion molecules, was shown by a radiolabeling technique to be constitutively expressed by bone marrow reticular cells as well as the entire endothelium of the bone marrow sinusoids [78]. Its counter receptor, VLA-4 ($\alpha_4\beta_1$), and ICAM-1, which belongs to a similar family and binds $\alpha_2\beta_1$ integrins, are found on many cancer cells. Thus adhesion molecules which serve normal bone metabolism can be used to the advantage of metastatic tumor cells.

Another integrin member, $\alpha_v\beta_3$ is associated both with breast cancer [79] and osteoblast function [80, 81]. Interestingly, it is over-expressed in metastatic breast cancer cells once they enter the bone [82]. It is the predominant integrin on osteoclasts and appears to be important for syncytia formation and attaching to the bone matrix [83]. Peptomimetic inhibitors of $\alpha_v\beta_3$ were found to significantly reduce metastatic cancer formation when injected prior to tumor cells in a mouse model. However, there was less of an effect when administered after tumor inoculation [84]. The expression of adhesion molecules by osteoclasts has been fairly well determined [85]. Three integrins, $\alpha_v\beta_3$, $\alpha_2\beta_1$, and $\alpha_v\beta_1$, and CD44 are present on osteoclasts.

The survival of cancer cells in the bone depends on their interactions with other cells. Interactions may be physical, with cell adhesion molecules, or through secreted molecules, such cytokines, chemokines, and other growth factors. Adhesion to various cells in the metastatic site

controls anti-apoptotic and proliferative signals (see [84]). Thus the bone marrow displays numerous adhesion molecules that offer opportunities for interactions between cancer cells and normal cells. Some of these interactions do not occur until the cancer cells are in the bone marrow environment after they express new adhesion molecules.

9 Bone remodeling and inflammation

Rodan [86] in an overview of skeletal development and function, points out the similarities between bone remodeling and inflammation. Many of the same cytokines produced by the immune cells as part of an inflammatory response are also produced by osteoblasts. Some of these, IL-1, IL-11, IL-6, PGE and PTHrP, are also osteoclastogenic. Furthermore, both osteoblasts and osteoclasts express toll-like-receptors [87] and respond to trauma, bacterial infection, and metastases with the production of these same molecules [88–90]. In particular, a set of inflammatory stress molecules (IL-6, IL-8, MCP-1, COX-2) appears in normal bone remodeling as well as under these pathogenic conditions [88]. These factors are made by osteoblasts but can also be produced by macrophages. They attract and activate osteoclasts. Osteoclasts degrade bone matrix, leading to the release of many growth factors. This combination of factors creates a very hospitable environment for cancer cells.

10 Cytokines and chemokines

Once established in the bone microenvironment, a 'vicious cycle' is created among metastatic tumor cells, osteoblasts, and osteoclasts that facilitates increased bone turnover and metastatic cell survival. Guise et al. developed a model of breast cancer metastasis to the bone, based on breast cancer cell overproduction of PTHrP [3, 91] that activates osteoblasts to produce RANK-L. Osteoblast-secreted RANK-L binds the RANK receptor on osteoclasts, inducing osteoclast differentiation and bone matrix degradation. In turn, TGF-β, released from the bone matrix, stimulates the cancer cells to produce more PTHrP [43], thus establishing a positive feedback loop. There is additional evidence that breast cancer-derived IL-8 acts prior to PTHrP to stimulate osteoclastogenesis via both RANK-L dependent and independent mechanisms [92–94]. As a result of constituitive osteoclast activation and an inability of osteoblasts to lay down bone matrix, sustained bone degradation occurs [45, 54]. This feedback establishes a vicious cycle, resulting in continued activation of osteoclasts and breast cancer cells. Ultimately, osteolytic lesions are formed at sites of metastases [10, 57].

It should be noted that the presence of PTHrP is not sufficient for cancer cell metastases to the bone. In a study in which 526 patients with operable breast cancer were examined, it was found that those with PTHrP-positive primary tumors had improved survival and were less likely to develop bone metastases [95]. In those patients with bone metastases, PTHrP presence was found not to be significantly associated with tumor size, vascular invasion, or tumor grade [95]. Thus, it is likely that bone metastases are influenced by other factors in the bone microenvironment besides PTHrP.

In addition to the PTHrP, Bendre et al. found an important role for IL-8. IL-8, the human homolog to murine MIP-2, belongs to the family of CXC chemokines and is naturally constitutively produced by osteoblasts [93, 96, 97]. IL-8 is overexpressed in a bone-homing derivative of MDA-MB-231 human metastatic breast cancer cells suggesting an important role in bone metastasis [94]. IL-8 can stimulate osteoclastogenesis by increasing RANK-L or stimulate the formation of osteoclasts in the absence of RANK-L [92]. It is believed that IL-8 is involved in the early stages of breast cancer metastasis by initiating the bone resorption process [93]. IL-8 also has been shown to increase angiogenesis and suppress osteoblast activity [98, 99]. In addition, IL-8 increases cell motility, invasion, and metastatic potential in breast cancer [93]. If overexpressed in breast cancer cells, IL-8 will lead to increased bone metastasis and osteolytic activity [94]. IL-8 stimulates osteoclast activity independently of RANK-L [92]. Bendre et al. suggested that the vicious cycle with PTHrP is first initiated by breast cancer cells secreting IL-8, thereby stimulating bone resorption by osteoclasts. The release of TGF-β from the bone matrix then stimulates cancer cells to produce more PTHrP, thus continuing the vicious cycle [93].

COX-2 and PGE_2 also have been found to contribute to osteoclast activitation and facilitate the creation of a microenvironment favorable for cancer cell metastasis. COX-2 levels and activity correlate with cancer cell metastasis both *in vitro* and *in vivo* [100–102]. COX-2 expression also has been implicated in the growth, invasion, apoptosis, and angiogenesis of breast cancer [103–105]. COX-2 expression in patients with cancer has shown to be a negative prognostic factor [106]. Singh et al. recently conducted a study investigating the involvement of COX-2 in breast cancer metastases to the bone [107]. Interestingly, overexpression of COX-2 correlated with increased production of IL-8 [108], which has also been linked to increased metastatic occurrence [94]. Singh et al. found that COX-2 induced both the formation of PGE_2 and IL-8 specifically in bone metastatic breast cancer cells. Since PGE_2 and IL-8 are mediators of osteoclast activation [109] (through direct or indirect mechanisms of stimulation of RANKL [92]), a system in which there is overexpression of

COX-2 would favor osteolytic cancer metastases. In addition, Hiraga et al. discovered that bone-derived TGF-β stimulated COX-2 expression, thus enhancing bone metastases in breast cancer [110]. TGF-β, released from the bone during bone resorption, stimulates COX-2 expression and subsequently PGE_2 expression in breast cancer cells. PGE_2 upregulates RANKL expression on osteoblasts, leading to osteoclast activation and increased bone turnover [110]. Finally, Hall et al. investigated the involvement of Wnts, a family of glycoproteins [111], in the promotion of osteoblastic bone metastases in prostate cancer [112]. They found that promotion of Wnt activity (by blocking the Wnt antagonist DKK-1), led to enhanced osteoblastic bone metastases in typically osteolytic PC-3 prostate cancer cells [112]. These results suggest that the involvement of DKK-1 dictates whether bone metastases are osteoblastic or osteolytic, and once again emphasize the importance of the bone microenvironment.

Cancer cell secreted IL-1, IL-6, and IL-11 have also been found to increase osteoclast activation. IL-6 is a pleiotropic cytokine that is naturally expressed by osteoblasts in low quantities. IL-6 receptors are found on osteoclasts and when stimulated, cause osteoclast differentiation and bone resorption [113]. There is a correlation of poor prognosis with increased IL-6 expression and metastatic breast cancer. IL-6 additionally has been implicated with increased breast cancer cell migration [114–116]. IL-1 is also a potent stimulator of osteoclast activation. To explore the notion that increased bone turnover attracts metastatic cancer cells, Sasaki et al. increased bone resorption by injecting recombinant IL-1β locally over the calvaria of nude mice [117]. Four weeks after cancer cell inoculation, osteolytic metastatic cancer cells were found in the calvariae of IL-1 treated mice. None were seen in the control. IL-11 is an additional key player in osteoclast activation. It has been reported that IL-11 mediates the actions of IL-1 on osteoclast development [118]; however, IL-11 has independent effects on osteoclast activity [119]. IL-11, IL-1, and IL-6 have all been found to be involved in an interacting cascade of cytokines which play a large part in osteoclast development and activity. Increased osteoclast activity subsequently creates a bone microenvironment that favors cancer cell metastasis, growth, and development. IL-1 contributes to the production of IL-11 and IL-6 [118]. IL-6 and IL-11 production are also regulated by IL-1, growth factors such as PDGF, IGF-1, and TGF-β, vitamin D, PTHrP, and PTH [119, 120].

11 Bone matrix is fertile soil for metastatic cancer cells

During bone deposition, osteoblasts secrete a variety of growth factors, such as IGF, TGF-β, FGF, and BMPs, that become incorporated into the bone matrix [121]. As bone resorption occurs, these factors are released into the bone microenvironment, making it an attractive place for cancer cells to metastasize and grow [122]. TGF-β released from the bone, in particular, has been found to stimulate the expression of CTGF, IL-11, and PTHrP by cancer cells [42, 123]. These factors are involved in tumor metastasis to bone and subsequently promote additional bone resorption, leading to the release of more growth factors and further preferential tumor metastasis to bone. Growth factors released during bone remodeling and present in the bone microenvironment may be chemoattractive molecules for the cancer cells. Orr et al. and Mundy et al. using a Boyden Chamber assay both demonstrated that the release of growth factors during bone resorption stimulated the chemotaxis of cancer cells [124, 125]. Additionally, cytokines have been implicated in cancer cell chemotaxis to bone. The SDF-1/CXCR4 axis is a ubiquitous chemotaxis mechanism in normal biology and is used for directed migration of a variety of immune and hematopoetic cells [126–129]. Jung et al. found that SDF-1 is secreted by osteoblasts, and that certain factors, namely IL-1β, PDGF-BB, VEGF, tumor necrosis factor-alpha (TNF-α), and PTH, act on osteoblasts to increase SDF-1 production [130]. Consequently, many of these cytokines play a role in increasing osteoclast activity during bone resorption [119, 120]. Furthermore, the results of this study suggested that osteoblast secretion of SDF-1 may be a chemotactic mechanism for stem cell homing. It goes without saying that the SDF-1/CXCR4 axis may be involved in cancer cell chemoattraction as well. In fact, Muller et al., among others [131, 132], explored the idea that metastatic breast cancer cells were responsive to gradients of chemokines [133]. CXCR4 was found to be highly expressed on metastatic breast cancer cells, and its ligand, SDF-1 was found to be highly expressed in organs to which cancer cells preferentially metastasizes (such as bone) [133]. Treatment with a neutralizing antibody to CXCR4 suppressed bone metastatic breast cancer [133]. Sun et al. conducted a similar study using prostate cancer as a model and found comparable results [134]. Once in bone, cancer cells become tethered by integrins and cell adhesion molecules as previously described [135, 136].

Current models suggest that chemokines and cytokines produced by breast cancer cells are key to breast cancer cell metastasis [92–94, 137]. Elevated levels of IL-8 production by human breast cancer cells have been correlated with increased bone metastasis *in vivo* and with stimulation of osteoclast differentiation and bone resorption [92, 94]. Tumor-derived IL-1, IL-6, and IL-11, insulin-growth factor-II, TNF-α, and a variety of other factors can also contribute to osteoclast activation and bone destruction [98, 138].

While breast cancer cells undoubtedly play an important role in breast cancer metastasis to the bone, data have shown that osteoblasts can be directed by the breast cancer cells to produce several inflammatory cytokines that have been implicated in osteoclast activation as well as breast cancer cell migration and survival [93, 139–143]. (Table 1 gives a brief summary of some relevant cytokines and their sources in the bone.) Kinder et al. demonstrated that osteoblasts undergo an inflammatory stress response in the presence of human metastatic breast cancer cells and produce elevated levels of IL-6, human IL-8 (murine KC, MIP-2), and MCP-1 [144]. These cytokines are known to attract, differentiate, and activate osteoclasts; thus co-opting osteoblasts into creating a bone microenvironment that exacerbates bone loss [144]. Similar findings were seen with murine osteoblasts and primary calvarial osteoblasts [144]. These results support the idea that cancer metastases create a unique niche in the bone microenvironment by co-opting normal cells of the bone to favor tumor growth and development.

Furthermore, Mastro and colleagues have preliminary *in vivo* evidence that osteoblasts themselves in the bone naturally produce cytokines that may be chemoattractants for metastatic breast cancer cells. In particular, they showed that metaphyses of bone cleared of bone marrow produced chemokines and cytokines that were different from those in the diaphysis (shaft). Prominent among these were KC (present only in the metaphyses), MIP-2 (murine homolog to human IL-8), and MCP-1 (Fig. 2) (Bussard and Mastro, 2007, unpublished data).

These cytokines were strongly observed in cultures of the bone metaphysis alone and not found in cultures of bone marrow from the metaphysis (Bussard and Mastro, 2007, unpublished data). This observation suggests that the cytokines were specifically produced by the cells of the bone (i.e. osteoblasts) and not the stromal cells. Murine IL-6, KC, and MIP-2 located in femur metaphyses were also found to be increased in the presence of human metastatic breast cancer cells compared with femur metaphyses from control mice (Bussard and Mastro, 2007, unpublished data). Finally, a novel experiment was conducted to monitor and quantify the initial stages (arrival, localization, and initial colonization) of breast cancer cell trafficking in the bone [59]. The DNA from femurs of mice inoculated with MDA-MB-435^{GFP} cells via intracardiac injection were isolated at various times, purified, and subjected to quantitative PCR for a human gene, HERV-1, and the number of breast cancer cells calculated. Femurs were separated into metaphyses and diaphyses. Results indicated that breast cancer cells preferentially migrated within days directly to the distal then proximal metaphyses. Few were found in the diaphyses [59]. These results additionally support the idea that metastatic breast cancer cells may follow a gradient of chemoattractant cytokines as well as suggests the importance of the local bone microenvironment.

In addition to IL-6 and IL-8, KC and MCP-1 are osteoblast-derived cytokines that greatly increase in response to metastatic breast cancer cells (Bussard and Mastro, 2007, unpublished data). MCP-1, a member of the CC chemokine family, is naturally produced by osteoblasts [96]. It regulates bone resorption by stimulating the migration of common monocyte-osteoclast progenitor cells from the blood or the bone marrow to the bone. MCP-1 concentrations are increased in metastatic cell lines, and it is associated with angiogenesis and increased cancer cell survival [145–147]. KC is another member of the CXC chemokine family with homology to IL-8 [148]. KC stimulates angiogenesis and is involved in neutrophil chemotaxis and activation [148, 149]. KC is also expressed by osteoblasts [149].

In addition to directing osteoblasts to secrete cytokines which alter the bone microenvironment, cancer cells affect the bone building cells in other ways as well. Mercer et al. demonstrated that culturing mouse osteoblasts with the conditioned medium from a human metastatic breast cancer cell line inhibited expression of osteoblast differentiation and blocked osteoblast ability to mineralize bone matrix [45]. This *in vitro* observation was confirmed in a mouse study [59]. Since osteoblasts do not differentiate properly in the presence of breast cancer cells, it is possible that the

Table 1 Source of several important chemokines and cytokines in the bone microenvironment in the presence of metastatic breast cancer cells

	Osteoblasts	Bone matrix	Breast cancer cells	Other cells in the bone microenvironment
IL-6	[153, 154]	–	[138, 155]	Bone Marrow Stromal Cells [156], Monocytes, Macrophages [157], and Osteoclasts [158]
IL-8	[155, 159]	–	[94, 155, 160]	Osteoclasts [155, 161], Bone Marrow Stromal Cells [159], Macrophages [162], Endothelial Cells [162]
PTHrP	–	–	[91, 94, 155]	–
TGFβ	[121, 155]	[155, 163]	[155]	Bone Marrow Stromal Cells [164], Endothelial Cells [165]

Many cells in the metastatic bone environment produce cytokines and chemokines. The cell sources of several important ones are indicated. The dash indicates that we were unable to find evidence from the literature or in our laboratory that the molecule was produced in the indicated cell type.

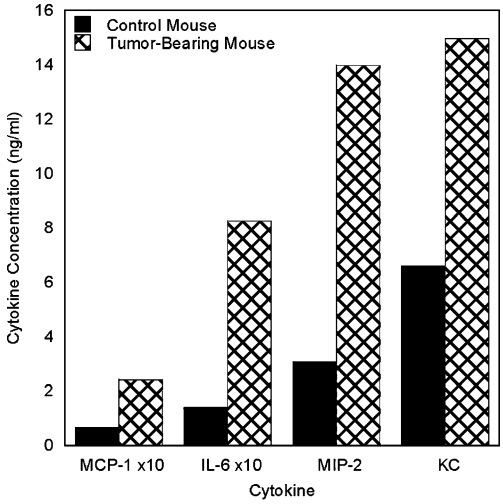

Fig. 2 Cytokine expression of murine femur metaphyses ex-vivo following intracardiac inoculation. MDA-MB-231^{GFP} cells (3×10^5 cells) were inoculated into the left cardiac ventricle of 4–6 week old athymic, female mice. Control mice were untreated. Mice were euthanized at 4 weeks and femurs harvested. The bone marrow was removed and the femur metaphyses were fractionated. Isolated metasphyseal bone pieces were crushed and cultured. Media were collected and tested after 24 h. Murine MCP-1 cytokine production was quantified using ELISAs. Murine IL-6, MIP-2, and KC were quantified using a Bio-Rad Bio-Plex™ murine cytokine quantification assay. Shown is a representative experiment. MCP-1 and IL-6 concentrations were multiplied by 10 in order to be shown on the same graph as MIP-2 and KC

cancer cells may alter the overall protein secretion profile of osteoblasts. This alteration may involve preventing osteoblasts from producing the differentiation proteins necessary for developing into mature, bone-depositing cells, as well as inducing osteoblast production of cytokines that could contribute to progression of bone metastasis, increase activation of osteoclasts, and contribute to the formation of osteolytic lesions.

12 Conclusions

Clearly, the organ microenvironment is extremely influential in cancer cell metastasis to a specific location. Crosstalk between the cancer cell "seed" and the target organ microenvironment "soil" will determine if the cancer cell metastasizes to a specific site and if that microenvironment supports growth and proliferation of the metastatic cancer cell. Only then will the metastatic cancer cell population flourish. Bone provides an especially attractive site for a variety of reasons. Metabolically active areas of bone are well-vascularized with a system that allows various cells to easily enter and exit. The normal remodeling process provides chemotactic and growth factors that attract cancer cells and support them once in place. The bone matrix contains a rich storehouse of growth factors such as TGF-β that are released during bone turnover. Resident cancer cells thrive in the rich cocktail of released cytokines. Finally, both osteoblast and octeoclast activities can be modulated by cancer cells to their advantage. The release of characteristic sets of cytokines by the bone matrix of an osteolytic lesion or osteoblastic lesion (e.g. MCP-1, IL-6, IL-8) will facilitate the chemoattraction and survival of metastatic cancer cells. Understanding the mechanisms behind these events will aid in the development of therapeutics to combat specific metastases and manipulate their target organ microenvironments. While the origin of metastatic variants remains unclear, is it certain that the target organ microenvironment contributes greatly to their metastasis.

13 Unanswered questions

Many unanswered questions remain. One of the most critical is to determine a 'metastatic signature' for the primary tumor which would indicate the possibility of metastasis. However, not all tumor cells that arrive in the bone, even from the same primary tumor, will remain or grow there. Dormant metastases in the bone remain a mystery. It is known that individual cells or micrometastases can be found in patients with no evidence of metastasis [150]. These individuals may never exhibit bone metastasis. On the other hand, bone micrometastases can remain dormant for years in spite of the rich microenvironment. What event triggers that cell to begin to grow?

14 Future studies

It is difficult to study bone metastases and the tumor microenvironment for many reasons. (1) The marrow space is relatively inaccessible. (2) It is also a complex space containing not only bone cells, osteoblasts and osteoclasts, but also hematopoietic cells and transient immune cells. Cell lines, particularly osteoblasts, have been developed that recapitulate in culture the stages of osteoblast differentiation. However, these lines have limitations when compared to intact bone.

We have recently developed a specialized bioreactor that allows extended-term culture of osteoblasts. The cells have been grown uninterrupted for up to 10 months. They proliferate and form a multilayer (>6 cell layers) of mature osteoblasts that begin to mineralize and form macroscopic bone chips [151]. By 10 months the morphology of the cells resembles osteocytes. We have inoculated human metastatic breast cancer cells, MDA-MB-231^{GFP}, into these chambers and have seen by microscopy that the cells adhere, grow, and move through the cell layers, mimicking *in vivo* migration and invasion [152]. We have evidence

that the osteoblasts likewise undergo a stress response and produce increased amounts of IL-6, for example. While the bioreactor has been used to study osteoblast-cancer cell interactions, it will allow introduction of other cell types, e.g. macrophages, lymphocytes. Thus, the bioreactor promises to be a useful 3-D culture system to study and to manipulate the bone-tumor microenvironment.

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Osteoblasts Naturally Produce Cytokines that Influence the Tumor Microenvironment in Bone Metastastic Breast Cancer, Karen M. Bussard* and Andrea M. Mastro, The Pennsylvania State University, University Park, PA, 16802

Breast cancer has a high propensity for bone colonization and specifically metastasizes to the ends of long bones in humans. Although the precise mechanism underlying preferential metastasis is unknown, it is likely that the bone (femur metaphyses) provides a hospitable environment that both attracts breast cancer cells and allows them to colonize and grow.

Bone remodeling begins when bone resorbing osteoclasts excavate an erosion cavity in the matrix. Next, bone depositing osteoblasts migrate to the cavity and synthesize layers of osteoid matrix that form new bone. Bone metastatic breast cancer cells disrupt the balance between osteoblasts and osteoclasts. Metastatic breast cancer cells induce osteoclast bone resorption, halt osteoblast deposition, and profoundly alter osteoblast properties. Current models implicate chemokines and cytokines produced by breast cancer cells as keys to understanding breast cancer cell metastasis. While cancer-derived cytokines undoubtedlyportant role, we have evidence that *osteoblasts* can be directed by metastatic breast cancer cells to produce inflammatory cytokines that may be chemoattractants, growth, or maintenance factors for the cancer cells as well as for osteoclasts.

We hypothesize that *osteoblast*-derived cytokines are increased in the presence of metastatic breast cancer cells and act as chemoattractants, growth, and maintenance factors for them. To further investigate these phenomanae, the current study was carried out **1) to determine how *osteoblast*-derived inflammatory cytokine production by MC3T3-E1 osteoblasts is altered in response to conditioned medium of bone metastatic MDA-MB-231 breast cancer cell variants.** The cytokine response of MC3T3-E1 murine osteoblasts to human metastatic breast cancer conditioned medium was examined using Raybio® murine cytokine arrays and quantified with either murine ELISAs or Bio-rad Bio-plex™ murine cytokine assays. Osteoblasts were cultured *in-vitro* for 4 (growth), 10 (early differentiation), and 20 days (late differentiation) and treated with either 0, 10, 25, or 50% conditioned media from human non-metastatic cells (hTERT-HME1 or MDA-MB-231BRMS) or human metastatic breast cancer MDA-MB-231 cell variants (parental MDA-231W, parental MDA-231PY, bone-seeking MDA-231BO, or brain-seeking MDA-231BR). Twenty-four hours later, culture supernatants were collected and quantified.

Cultured MC3T3-E1 osteoblasts increased production of IL-6, MCP-1, and VEGF in the presence of metastatic breast cancer cell conditioned media as determined by a Raybio® murine cytokine screen. An increase in murine KC was detected using a Bio-rad Bio-plex™ murine cytokine assay. KC, MCP-1, and IL-6 were secreted in a dose-response manner with increasing conditioned medium treatment. The largest induction of osteoblast-derived cytokine production occurred in 20 day old osteoblasts (late differentiation). Treatment with the conditioned medium of a MDA-231 bone-seeking variant further enhanced osteoblast cytokine secretion at day 20. Furthermore, osteoblast-derived MCP-1 expression varied dramatically with conditioned media treatment. MCP-1 expression was not detected in osteoblasts treated with conditioned media from non-metastatic human mammary epithelial cells, but was significantly

increased in osteoblasts treated with metastatic breast cancer conditioned media. Collectively, these results suggest that osteoblasts undergo an inflammatory response in the presence of metastatic breast cancer cell conditioned medium.

In addition, we sought **2) to determine how bone-derived inflammatory cytokine production is altered in response to breast cancer cells *in vivo*.** Femurs from either control or mice inoculated via intracardiac injection with metastatic breast cancer cells were assayed *ex vivo* for breast cancer cell trafficking patterns and inflammatory cytokine production using quantitative PCR and species-specific antibody arrays respectively. The metaphyses (ends) of femurs cleared of bone marrow expressed a different pattern of cytokines than the diaphysis (shaft). In particular, it was found that metaphyses of bone in normal mice cleared of bone marrow produced substantial amounts of KC, MIP-2, and MCP-1. In mice inoculated with MDA-MB-231W human metastatic breast cancer cells, the breast cancer cells preferentially trafficked to the metaphyses of the bone as opposed to the diaphysis, where concentrations of MCP-1, IL-6, MIP-2, and KC increased significantly from control values as determined by standard ELISAs and Bio-rad Bio-plex™ murine cytokine assays. Taken together, these results suggest that metastatic cancer cells are attracted to cytokines naturally secreted by osteoblasts that are concentrated in the ends of the femur.

In summary, we propose that metastatic breast cancer cells are attracted to cytokines naturally produced by osteoblasts. Once in the bone microenvironment, metastatic breast cancer cells induce osteoblasts to undergo a stress response, increasing osteoblast production of these chemotactic inflammatory cytokines.

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AACR 2008 Abstract
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Osteoblast-Derived Cytokines are Major Mediators in Facilitating in Bone Metastatic Breast Cancer

Breast cancer (BC), with its predilection for bone metastases, is the second leading cause of cancer deaths in American women. While the mechanism for directional metastasis is unknown, the bone microenvironment likely provides a fertile soil for metastatic BC cells. Besides affecting osteoblast (OB) and osteoclast (OC) properties, we have evidence that metastatic BC cells further create a unique bone niche by co-opting *osteoblasts* to increase production of inflammatory cytokines that may be chemoattractants, growth, or maintenance factors for cancer cells or OCs.

MC3T3-E1 murine OBs were grown to various stages of maturity: 4 (growth), 10 (early differentiation), and 20 days (late differentiation) and incubated with conditioned medium (CM) from human metastatic BC MDA-MB-231 cell variants (parental MDA-231W, parental MDA-231PY, bone-seeking MDA-231BO, or brain-seeking MDA-231BR). Culture supernatants were assayed for cytokine expression with species-specific Bio-Rad Bio-Plex™ cytokine arrays. Also, femurs from mice inoculated via intracardic injection with MDA-231-GFP BC cells were assayed *ex vivo* for cytokine production.

MC3T3-E1 murine OBs treated with human metastatic BC CM produced increased amounts of murine IL-6, VEGF, MIP-2 (human IL-8), KC (human GRO-α), and MCP-1 with the largest induction seen in 20 day old OBs. The human metastatic BC cell variants themselves produced a similar array of cytokines: IL-6, VEGF, IL-8, and GRO-α. However, the human metastatic BC cell variants produced only very small amounts of MCP-1. Monocyte chemoattractant protein-1 (MCP-1) is associated with angiogenesis and increased cancer cell survival. MCP-1 also regulates bone resorption by stimulating the migration of monocyte-osteoclast progenitor cells to the bone.

These same cytokines were detected *ex vivo* in femurs of mice bearing human metastatic bone metastases. It is known that MDA-231 BC cells preferentially traffic to the metaphyseal ends of long bones. We found that metaphyses of femurs cleared of bone marrow expressed cytokines different from those in the diaphysis. Furthermore, concentrations of IL-6, VEGF, KC, MIP-2, and MCP-1 increased significantly in cancer-bearing mice compared to non-cancer-bearing mice.

Overall, these data suggest that OBs are an important source of cytokines, specifically MCP-1, in BC bone metastasis. The nature of this cytokine panel suggests their importance for the attraction and activation of OCs leading to increased bone resorption in bone metastatic BC. Thus, these findings clearly implicate the bone microenvironment and cancer cell manipulation thereof in facilitating metastatic tumor cell colonization and survival.

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EDUCATION

The Pennsylvania State University, University Park, PA
Ph.D. in Pathobiology Date expected: Early Fall 2008
Ph.D. G.P.A. 3.95
Ph.D. thesis presently entitled "The Role of Osteoblast-Derived Inflammatory Cytokines in Bone Metastatic Breast Cancer"

The Pennsylvania State University, Hershey, PA
M.S. in Biomedical Engineering 2000-2002
Master's thesis entitled "Interactions of Model Biomaterials and Enzymes in Contact Activation of the Blood Plasma Coagulation Cascade"

Fairleigh Dickinson University, Madison, NJ
B.S. in Biology / minor in Chemistry 1995-1999
Honor's thesis entitled "Sports Injuries: How They Occur, Methods of Treatment, and Ways of Prevention"

WORK EXPERIENCE

Graduate Research Assistant 8/2004-present
The Pennsylvania State University, University Park, Pennsylvania
Advisor: Andrea M. Mastro, Ph.D., Professor of Microbiology and Cell Biology
- Investigate the influence of osteoblast-dervied inflammatory cytokines on metastatic breast cancer cells.
- Perform intracardiac inoculations on athymic nude mice.
- Study and quantify the trafficking of breast cancer cells throughout long bones.
- Develop and perform protocols and analysis.
- Supervise, educate, and orient students in performance of protocols.

Research Support Associate/Graduate Research Assistant 8/2000 – 8/2004
The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania
Advisor: Christopher A. Siedlecki, Ph.D,. Assistant Professor of Surgery and Bioengineering
- Study adhesion of blood coagulation factors to biomaterials with Atomic Force Microscope.
- Investigate the clotting mechanisms of human plasma in response to biomaterials.
- Analyze bovine blood of Left Ventricular Assist Device and Total Artificial Heart recipient research calves with the Thromboelastograph Hemostasis Analyzer (TEG).
- Develop and perform protocols and analysis.
- Supervise, educate, and orient assistant in performance of protocols.

Laboratory Technician II 10/1999 – 8/2000
University of Maryland Medical System, Baltimore, Maryland
- Blood Bank Technician assisting Medical Technologists in their work.
- Issue and perform ABO checks on blood products, receive, record, and sort blood specimens.
- Assist physicians in clinical decision making on product administration.
- Irradiate and thaw blood products.
- Cancer Center Platelet Laboratory Technician assisting physicians in clinical decision making of product administration and providing patients with platelets.

Laboratory Technician 10/1997 – 5/1999
Severn Trent Laboratories, Whippany, New Jersey
- Inorganics Laboratory Technician testing samples for Biochemical Oxygen Demand (BOD), Phenol distillation and reading, Alkalinity, Toxicity Characteristic Leaching Procedure (TCLP), ZHE tumbling and leaching, Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Total Solids (TS), Reactivity, Cyanide, Specific Gravity, and Ignitability.
- Made reagents necessary for and calibrated instruments used for each test.

PROFESSIONAL MEMBERSHIPS

2002-2003	Society for Biomaterials
2003	American Society for Artificial Internal Organs
2005-present	Sigma Xi, The Scientific Research Society
2005-present	American Association for Cancer Research
2005-present	Women in Cancer Research (WICR)

PUBLICATIONS

2001 Siedlecki, CA, **Bussard, KM**, Vogler, EA. "FXII interactions with biomaterial surfaces." American Society for Artificial Internal Organs Journal. 47: (2), 170.

2005 Zhuo, R, Miller, R, **Bussard, KM**, Siedlecki, CA, Vogler, EA. "Procoagulant Stimulus Processing by the Intrinsic Pathway of Blood Plasma Coagulation." Biomaterials. 26: (16), 2965.

2005 Guo, Z, **Bussard, KM**, Chatterjee, K, Miller, R, Vogler, EA, Siedlecki, CA. "Mathematical Modeling of Material- Induced Blood Plasma Coagulation." Biomaterials. 27: (5), 796.

2006 Phadke, PA, Mercer, RR, Harms JF, Jia, Y, Kappes, JC, Frost, AR, Jewell, JL, **Bussard, KM,** Nelson, S, Moore, C, Gay, CV, Mastro, AM, Welch, DR. "Kinetics of Metastatic Breast Cancer Cell Trafficking in Bone." Clinical Cancer Research. 12: (5) 1431.

2008 Kinder, M, Chislock, EM, **Bussard, KM**, Shuman, LA, Mastro, AM. Metastatic Breast Cancer Induces an Osteoblast Inflammatory Response. Experimental Cell Research. 314: (1), 173.

2008 **Bussard, KM**, Gay, CV, Mastro, AM. The Microenvironment in Metastasis: What is Special About Bone? Accepted to Cancer Metastasis Reviews. Available on Epub ahead of print December 11, 2007.

2008 **Bussard, KM**, Mastro, AM. Osteoblast-derived MCP-1 Facilitates Bone Metastatic Breast Cancer. (working title) Manuscript in preparation.

ORAL PRESENTATIONS

2008 **Bussard, KM**, Mastro, AM. "Osteoblast-Derived Cytokines are Major Mediators in Facilitating Bone Metastatic Breast Cancer." Presented at the American Association for Cancer Research Annual Meeting's Tumor Biology Minisymposium, April 12-16, 2008.

2006 **Bussard, KM,** Phadke, PA, Mercer, RR, Harms JF, Jia, Y, Kappes, JC, Frost, AR, Jewell, JL, Nelson, S, Moore, C, Gay, CV, Mastro, AM, Welch, DR. "Kinetics of Metastatic Breast Cancer Cell Trafficking in Bone." Presented at the American Association for Cancer Research Annual Meeting's Tumor Biology Minisymposium, April 1-5, 2006.

POSTER PRESENTATIONS

2002 **Bussard, KM**, Vogler, EA, Siedlecki, CA. "Effective Production of FXIIa and Thrombin by Model Biomaterials." Proceedings of the IEEE 28th Annual Northeast Bioengineering Conference, 02CH37342, April 20-21, 2002

2002 **Bussard, KM**, Vogler, EA, Siedlecki, CA. "Effective Enzyme Production of FXIIa and Thrombin by Model Biomaterials." Transactions of the 28th Annual Society for Biomaterials Conference, Volume XXV, April 24-27, 2002.

2003 **Bussard, KM**, Trivedi, SH, Vogler, EA, Siedlecki, CA. "Surface Acceleration of Blood Plasma Coagulation." Transactions of the 29th Annual Society for Biomaterials Conference, Volume XXVI, April 30-May 3, 2003.

2003 **Bussard, KM**, Zapanta, CM, Rosenberg, G, Pae, WE, Siedlecki, CA. "Thromboelastograph Evaluation of Bovine Blood." American Society for Artificial Internal Organs Journal 49: (2) 222. Presented at the Annual ASAIO-ISAO Joint Conference, June 19-21, 2003.

2005 **Bussard, KM,** Shuman, LS, Mercer, RR, Phadke, PA, Nelson, SM, Jewell, JL, Chislock, EM, Kinder, M, Welch, DR, Gay, CV, Mastro, AM. "The Interaction of Metastatic Breast Cancer Cells with Osteoblasts." Presented at the CrossOver 2005 Meeting sponsored by The Huck Institutes of The Life Sciences and The Materials Research Institute, The Pennsylvania State University, October 13-14, 2005.

2006 **Bussard, KM,** Chislock, EM, Kinder, M, Gay, CV, Mastro, AM. "A Classic Set of Osteoblast-Derived Inflammatory Cytokines is Produced in Response to Bone Metastatic Breast Cancer." The 11 th International Congress of the Metastasis Research Society, Tokushima, Japan, September 3-6, 2006.

2007 **Bussard, KM**, Mastro, AM. "Osteoblast-derived Inflammatory Cytokines are Produced in Response to Human Metastatic Breast Cancer Cells." The 100th Annual American Association for Cancer Research Annual Meeting, Los Angeles, CA, April 14-18, 2007. Proceedings of the 97th Annual Meeting for American Association for Cancer Research, Volume 48.

2007 **Bussard, KM**, Mastro, AM. "Osteoblasts Naturally Produce Cytokines that Influence the Tumor Microenvironment in Bone Metastatic Breast Cancer." Skeletal Complications of Malignancy V, The Paget Foundation. October 25-27, 2007.

AWARDS AND HONORS

- American Association for Cancer Research – Scholar-In-Training Award: 2008
- The Pennsylvania State University College of Agricultural Sciences Travel Award: 2008.
- American Association for Cancer Research – Edward A. Smuckler "Pathobiology of Cancer" Workshop selected attendee. Snowmass, CO: July 15-22, 2007.
- American Association for Cancer Research – Women in Cancer Research Brigid G. Leventhal Scholar Award in Cancer Research: 2006.
- Department of Defense Predoctoral Traineeship Award in Breast Cancer (Fellowship): 2006-2009.
- The Pennsylvania Space Grant Consortium NASA Space Grant Fellowship: 2005-2007, 2007-2009.
- Sigma Xi Grants-in-Aid of Research: 2005-2006.
- Chancellor's List Publication: Inaugural Edition (2005), 2006.
- Honorable Mention for Student Travel and Professional Development Award, Society for Biomaterials: 2002.
- FDU Outstanding Academic Excellence Award in Biology: May 1999.
- FDU James A. Griffo Award for Academic Excellence and Athletic Achievement during college career: May 1999.
- FDU University Honors Program: 1995-1999 (Senior thesis entitled "Sports Injuries: How they occur, Methods of Treatment, and Ways of Prevention" presented April 30, 1999).
- FDU Presidential Scholar (Full Academic Tuition Scholarship): 1995-1999.
- Member: Phi Zeta Kappa (FDU Junior Honor Society, GPA 3.5+).
- Member: Phi Omega Epsilon (FDU Senior Honor Society, GPA 3.5+).
- Who's Who of Students in American Colleges and Universities Publication: 1995-1999.
- The National Dean's List Publication: 1995-1999.